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GENETIC CONCEPT FOR THE ORIGIN OF CANCER

BY

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GENETIC CONCEPT FOR THE ORIGIN OF CANCER*

Consulting Editor
LEONELL C. STRONG

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INTRODUCTORY REMARKS

By Leonell C. Strong

Biological Station, Roswell Park Memorial Institute, Springville, N. Y.

This monograph embodies three purposes: (1) to review the historical evelopment of the genetic concept for the origin of cancer, (2) to introduce ew observations in the genetics of cancer, and (3) to give an opportunity to her scientists using different techniques to report their findings in order to scertain whether these discoveries are complementary or antagonistic to a enetic concept.

The role of genetics in the study of cancer is twofold: (1) to investigate the ossibility of a host or constitutional influence on cancer susceptibility and ancer resistance, and (2) to visualize the intrinsic or cellular changes, in ontrast to the extrinsic or environmental influences, involved in the origin f the cancer cell from a somatic one.

The term intrinsic or cellular change is to be interpreted from the beginning n very general terms. Since there are several distinctive processes, such as norphogenesis and mutations, in cellular behaviors in normal physiology, all hese phenomena are considered in this publication. Beginning with so-called oint mutations and disturbances of the spacial relationships existing between enes by such changes as inversions, translocations, losses or additions of vhole chromosomes, many other changes also are discussed. Alterations in he cytoplasmic components are considered. This complex situation of bioogical forces is further resolved by the various contributors.

I hope that the availability of this material in a single monograph will nake possible a synthesis of the many and varied contributions bearing upon he origin of cancer.

GENETIC CONCEPT FOR THE ORIGIN OF CANCER: HISTORICAL REVIEW*

By Leonell C. Strong

Biological Station, Roswell Park Memorial Institute, Springville, N. Y.

The genetic concept for the origin of cancer is an old one. It has been used merely as a suggestion (Murray, 1908[48]) and only after the exclusion of other possibilities (Tyzzer, 1916[73]). Boveri,[12] however, anticipated these investigators by many years. Noting the presence of atypical cell divisions in embryonic and other tissues, he reasoned that these aberrant mitoses would lead to aberrations in the number of chromosomes and that some of these imbalanced cells, under certain conditions, might lead to aberrations in growth and hence to cancer, which is considered by many scientists to be a form of atypical growth. Boveri's concept has been ignored or neglected almost from its conception, but is now receiving consideration, particularly from the findings with the ascites tumors determined by George and Eva Klein[38-40] in Stockholm, Sweden, Yoshida[75] in Tokyo, Japan, Hauschka[28] in Buffalo, N. Y., Levan[44, 45] in Lund, Sweden, and many other investigators. In 1933, Henschen[32] published a brief review of the subject of the mutation concept, citing some of the earlier opinions of D. von Hansemann, Boveri, and A. P. Dustin.

The genetic concept for the mutation idea as a causative principle in the origin of cancer has been discussed, pro and con, by a number of investigators. Since cancer arises in somatic tissue, the attempt has been made to distinguish sharply between a somatic mutation and a germinal one.[7, 17] The distinction, however, is not an absolute one since, especially in plant material, noticeably in chrysanthemums, the ability of the plant to undergo somatic mutations is under gene control.[18] The major development in the mutation concept in relation to the origin of cancer was done, about the same time, by Bauer[7] in Germany and by myself[63, 64] in the United States. Bauer based his conclusion on a study of human material, whereas Strong was the first to obtain data on the transplantation of cancerous tissue in mice that could be interpreted in terms of somatic mutations (sudden changes in the growth capacities and histological characteristics of neoplastic tissues

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[*] The investigation reported in this paper has been aided in part by grants administered through Health Research, Inc., from the National Cancer Institute, Public Health Service, Bethesda, Md., and from The Committee on Growth of the National Research Council, Washington, D. C., acting for The American Cancer Society, New York, N. Y.

This manuscript was prepared in 1955 to serve as a series of lectures at the Karolinska Institute, Stockholm, Sweden. Advances in the fields of genetics, histocompatible genes, antigenicity, and immunity of transplantable tumors and polyploidy in reference to the cancer cell have been so rapid that both D. B. Amos and T. S. Hauschka, whose comments appear in this work, have broadened their opinions accordingly. For their latest interpretations see Amos's article "Genetic Studies on Tumor Immunity," elsewhere in this monograph, and their article "Cytogenic Aspects of Compatibility," in *Annals of The New York Academy of Sciences*, Volume 69, Article 4.

rpetuated by cell division from the point of origin and remaining stable r many generations).

The development of the original inbred strains of mice[65] such as the A, 3H, CBA made this contribution on the transplantable tumor possible. In 1926 it was discovered[63, 64] that the changes in the transplantability aracteristics of the cancerous tissue underwent sudden and unpredictable periodic changes that were relatively permanent from the point of origin (FIGURES 1, 2, and 3; TABLES 1, 2, and 3). Thus, highly specific tumors showed a very large number of genes in the constitution of the host that must be simultaneously present for the continued growth of the tumor. With time and sufficient numbers of suitable genetically controlled animals, a tumor showing a five Mendelian factor ratio thus changed to a derived tumor dependent upon four, then upon three, then upon two, then upon one, and

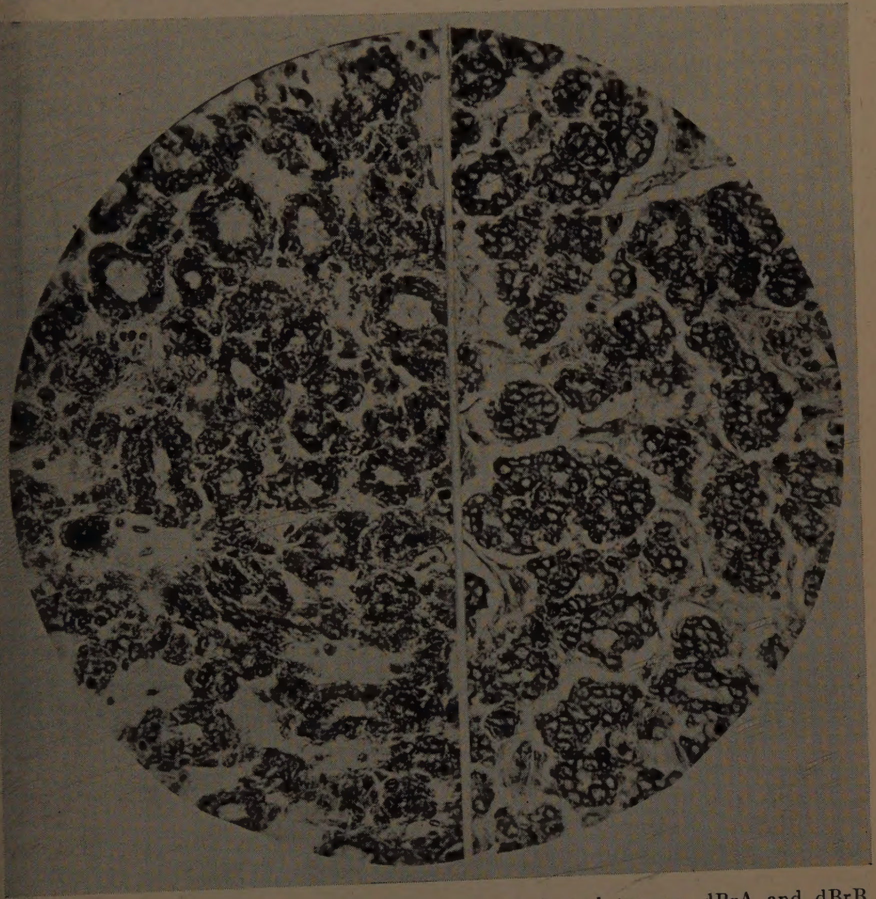

FIGURE 1. Histological comparison of two transplanted tumors, dBrA and dBrB. These tumors arose spontaneously in two mice of the D strain. The dBrA tumor showed a 3 Mendelian factor ratio, whereas dBrB gave a 2 factor ratio.

finally the tumor would grow in all mice of the F_2 generation where Mendelian segregation could no longer be measured. Up to the time when the transplant depended upon a single gene for its continued survival, the tumor would grow only in mice of the original inbred strain, in the F_1 of a cross between a mouse of a susceptible and a mouse of a resistant strain, and in a certain percentage of the F_2 population and the backcross generations, depending upon the number of genes responsible for progressive growth. When the tumor would grow in all F_2 mice then, and not until then, would it grow in all mice of all foreign strains, irrespective of genetic relationships. In other words, from a dependent type of growth the tumor had become progressively less nonspecific; these progressive changes could be measured in terms of fewer and fewer segregating genes responsible for the tumor's growth or survival.

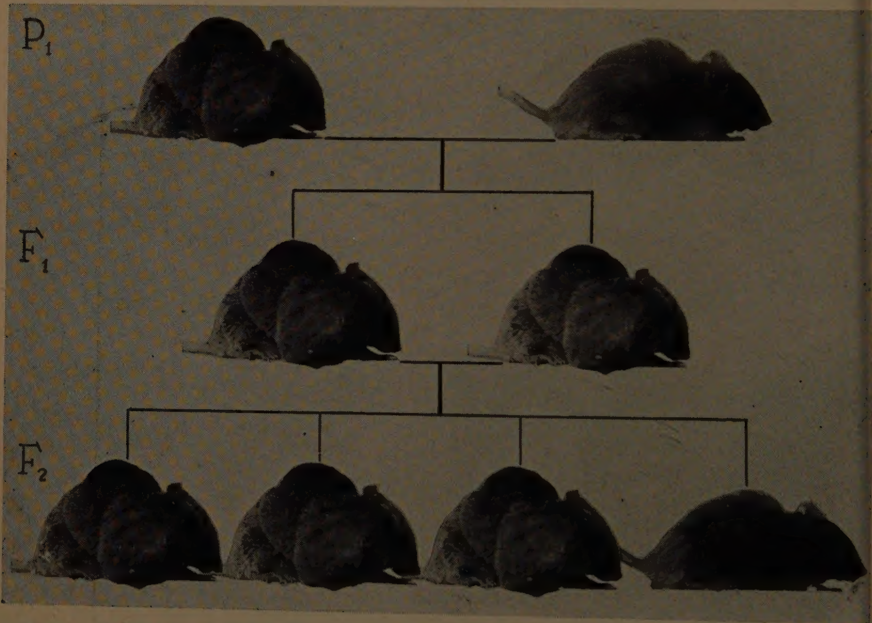

FIGURE 2. Schematic representation of the inheritance to a transplantable tumor such as dBrCap, which showed the influence of one gene on susceptibility.

The general conclusion drawn in 1926 was that the fate of the transplanted tumor was dependent upon a reaction between the host and the tumor cell. Both moieties of this reaction were determined by genetic constitutions. At that time it was considered that the genetic constitution of the tumor cell could not be confined exclusively to the gene or to the chromosome, but was considered to be all-inclusive in order to include the components of the cytoplasm. This early genetic concept, formulated in 1926, has been greatly exemplified since that time and is still of significance in the evaluation of data dealing with the origin of cancer.

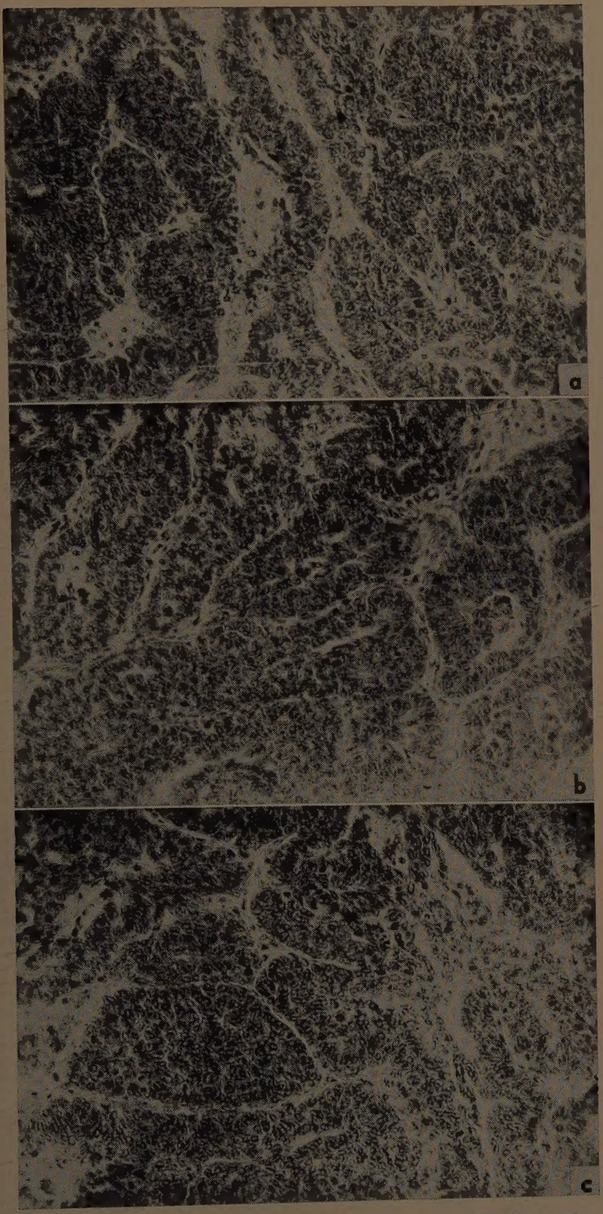

FIGURE 3. (a) Photomicrograph of the original tumor, dBrD. The tumor gave a 5 to 7 Mendelian factor ratio when inoculated into a series of F_2 individuals. (b) Tumor transplant (dBrDm) derived from the original tumor dBrD. Tumor dBrDm gave a 2 factor ratio when inoculated into the same series of F_2 individuals inoculated with the dBrD tumor. (c) Another tumor transplant (dBrDBs) derived also from the original tumor, dBrD, during the process of transplantation. This tumor showed a 1 factor ratio when inoculated into a similar series of F_2 individuals inoculated with tumor dBrD.

TABLE 1

DATA OBTAINED FOR THE THREE TRANSPLANTABLE TUMORS, dBrDm, dBrDBl, AND dBrDBs, IN THE F_2 GENERATION, TOGETHER WITH THE COMPARABLE DATA FOR THE ORIGINAL dBrD TUMOR

Experiment No.	Name of tumor	Equation	F_2 generation	Number of individuals	Per cent negatives	Difference between equations
1	Original dBrD	1	Observation	16.00+:96.00− ± 2.47	85.71% ± 2.20	
2	dBrDm	5	Observation	56.00+:43.00− ± 3.29	43.43% ± 3.32	1 and 5 = 42.28% ± 3.98 or 10.62 × PE.
		6	Expectation 1 factor	74.25+:24.75− ± 2.91	25.00% ± 2.94	5 and 6 = 18.43% ± 4.52 or 4.07 × PE.
		7	Expectation 2 factors	56.68+:43.31− ± 3.28	43.75% ± 3.31	5 and 7 = 0.32% ± 4.88 or 0.09 × PE.
		8	Expectation 3 factors	41.77+:57.23− ± 3.27	47.80% ± 2.88	5 and 8 = 14.37% ± 3.80 or 3.78 × PE.
3	dBrDBl	9	Observation	66.00+:56.00− ± 3.66	45.90% ± 3.00	1 and 9 = 39.81% ± 3.72 or 10.70 × PE.
		10	Expectation 1 factor	91.50+:30.50− ± 3.19	25.00% ± 2.61	9 and 10 = 20.90% ± 3.97 or 5.01 × PE.
		11	Expectation 2 factors	68.63+:53.37− ± 3.65	43.75% ± 2.99	9 and 11 = 2.15% ± 4.24 or 0.51 × PE.
		12	Expectation 3 factors	51.48+:70.52− ± 3.63	57.80% ± 2.98	9 and 12 = 11.90% ± 4.22 or 2.82 × PE.
4	dBrDBs	13	Observation	88.00+:36.00− ± 3.03	29.03% ± 2.44	1 and 13 = 56.68% ± 3.28 or 17.28 × PE.
		14	Expectation 1 factor	93.00+:31.00− ± 3.21	25.00% ± 2.42	13 and 14 = 4.03% ± 3.43 or 1.17 × PE.
		15	Expectation 2 factors	69.75+:54.25− ± 3.71	43.75% ± 3.00	13 and 15 = 14.72% ± 3.87 or 3.80 × PE.

TABLE 2

DATA OBTAINED FOR THE THREE TRANSPLANTABLE TUMORS, dBrCm, dBrCsp, AND dBrCX, IN THE F_2 GENERATION, TOGETHER WITH THE COMPARABLE DATA FOR THE ORIGINAL dBrC TUMOR

Name of tumor		F_2 generation	Number of individuals	Per cent negatives	Difference between
Original dBrC	1	Observation	23.00+:102.00− ± 2.89	81.60% ± 2.30	
dBrCm	5	Observation	99.00+: 65.00− ± 4.17	39.64% ± 2.53	1 and 5 = 41.96% ± 3.42 or 12.26 × PE.
	6	Expectation 1 factor	123.00+: 41.00− ± 3.55	25.00% ± 2.16	5 and 6 = 14.64% ± 3.32 or 4.41 × PE.
	7	Expectation 2 factors	92.25+: 71.75− ± 4.23	43.75% ± 2.57	5 and 7 = 4.11% ± 3.60 or 1.14 × PE.
	8	Expectation 3 factors	69.20+: 94.80− ± 3.60	57.81% ± 2.20	5 and 8 = 18.17% ± 3.35 or 5.42 × PE.
dBrCsp	9	Observation	75.00+: 23.00− ± 2.79	23.47% ± 2.86	1 and 9 = 58.13% ± 3.67 or 15.84 × PE.
	10	Expectation 1 factor	73.50+: 24.50− ± 2.85	25.00% ± 2.90	9 and 10 = 1.53% ± 4.07 or 0.37 × PE.
	11	Expectation 2 factors	55.10+: 42.90− ± 3.27	43.75% ± 3.36	9 and 11 = 20.28% ± 4.41 or 4.59 × PE.
dBrCX	12	Observation	243.00+: 2.00− ± 0.94	0.82% ± 0.38	1 and 12 = 80.78% ± 2.48 or 32.57 × PE.

Table 3

Tabulated Synopsis of the Various Transplantable Tumors Used in the Present Experiment, Showing the Probable Number of Independently Segregating Genetic Factors Involved in the Process of Transplantation

Name of tumor	Number of genetic factors
Original dBrC	6
dBrCm	2
dBrCsp	1
dBrCX	Undetermined

Confirmatory observations on the nature of the transplantable tumor were made by Bittner[10] and by Cloudman.[13] From this early work it was determined that no two tumors, even derived from the same mouse, ever gave the same Mendelian ratio of susceptible to resistant mice in the F_2. In the early transfer generations this is evidence of populations of cancer cells rather than pure cultures. With later transfer generations, after the process of serial or progessive "breakdowns" of susceptibility have occurred, some tumors have similar and perhaps identical genetic backgrounds. This will be made clear in the investigations of "isogenic" strains of mice. The early evidence on transplantation, then as well as now, is not intended to be conclusive, since the only method then available to prove a mutation was hybridization and, of course, there is nothing with which to hybridize a cancer cell. Nevertheless, it is clear that the sudden changes in growth capacities of transplantable tumors with the concomitant simpler genetic mechanism that occurs at exactly the same time is precisely what one would expect, provided a somatic mutation had occurred in a cancer cell. Changes in the host could not be responsible for the sudden changes in transplantable tumors, since comparisons were made in the same series of hosts.

However, with the development of serologic methods for the detection of antigens, this problem can now be reinvestigated. In relation to the tissue of origin and early transplant generations, perhaps it would be wise to use spontaneous tumors rather than the late transplantable tumors, where the process of transplantation may introduce some variables not necessarily involved in the origin of neoplastic tissue; this deviation is clearly cumulative with time.

Until very recent times the progressive increase of growth capacity and transplantability potentials of tumors has always occurred in one direction. However, recently both Hauschka[27] and Strong[70] have reported that this sequence of events is reversible. Thus, a relatively nonspecific tumor has become relatively more specific at the same time that an *increased* genetic complex for transplantation has occurred. Strong[69] reported that a transplantable tumor at one time depended upon either one of two genes for its successive growth (a 15:1 ratio in the F_2). Later the tumor changed and then required the simultaneous presence of both genes for its growth (9:7

tio in the F_2). For many years these suddenly appearing, relatively stable
permanent changes in the transplantable tumor have taken place without
demonstrable influence from the host. Recently it has been demonstrated
at these sudden changes in transplantation of cancerous tissue are not
rtuitous, but may be conditioned somehow by the host. In other words,
e transplantable tumor and changes in the transplantability of the tumor
e not completely autonomous. The evidence for this concept is as fol-
ws. Three strains of inbred mice have been used for this purpose. These
e the F, pBr, and N. The N strain was one of the ancestral stocks that
ve rise, by hybridization to the CBA and JK stocks, to the pBr. The F
rain is entirely independent in origin from the pBr and N strains. Follow-
g the subcutaneous injection of methylcholanthrene into mice of the pBr
train, a series of tumors of the forestomach have been obtained. One of
ese, a spindle-cell neoplasm,* has been continued subcutaneously in mice
f the pBr strain for more than 100 transfer generations. The tumor will
lso grow, although at a very reduced growth rate, in mice of the N strain.
t will not grow in mice of the F strain. The tumor will grow also in both
he F_1 generations, first in the cross between mice of the pBr × N strains
nd, second, in a cross between mice of the F and the pBr strains. Following
he transfer of the pBr tumor in mice of the N strain for varying lengths of
ime or transfer generations, the various tumors were then returned to mice
f the pBr strain. These derived tumors were then referred to as the
N-adapted, the 2N-adapted, the 3N-adapted, etc.

In the present experiment, 4472 mice have been grafted subcutaneously
vith a spindle-cell neoplasm of the forestomach. The transplantable tumor
as been divided into 4 subtransplants by the method of keeping the trans-
lant for a variable length of time in mice of the N strain. All 4 transplants
ave different genetic ratios when grafted into a series of F_2 individuals from
a cross of mice of the pBr and the N strains. The different ratios obtained
may be explained in terms of genic changes. However, it must be con-
cluded that these changes are not fortuitous, but may be dependent upon
some peculiarity of the host. The mechanism that determines transplant-
ability potentials of the tumor in mice of the pBr and N strains and their
derived hybrids can change without changes in the mechanism involved in
the growth or failure of the same tumor in mice of the F strain or its derived
hybrids.

The F_1 generation of a cross between mice of two distinct inbred strains
that differ in any one of a large number of physiological characteristics is now
being used for significant observations in a great variety of experiments;
much of the original genetic concept for the origin of cancer was based on the
F_1 generation of this cross. The value of such a technique is based upon the
finding that susceptibility to the transplantable tumor and other tissues was
demonstrated to be inherited as a dominant complex. Thus the F_1, being
a mosaic of potentialities derived from two ancestral stocks, is capable of
growing tumors from mice of each of the ancestral stocks used in the produc-

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* A recent review of the sections of this transplantable tumor by H. C. Stoll has led him to conclude that the tumor is an anaplastic carcinoma, which is pleomorphic.

tion of the F_1, as well as tumors arising in other F_1's. Mice of neither ancestral stock, however, were capable of growing tumors that arose in mice of the F_1 generation.

Kirschbaum[37] has used this technique in investigating susceptibility to leukemogenic agents such as X rays, methylcholanthrene, and estrogenic hormone. Thus, he has shown that sensitivity to each of these leukemogenic agents is under gene control, and the synergistic effect of two of these agents is also dependent upon a particular genic complex.

Law[41] has shown that only the thymus of a thymoleukemic-susceptible strain will give rise to leukemia when grown in the F_1. The thymus from a leukemia-resistant strain will fail to develop into leukemia even when grafted into the same individual. In five cases in which the original thymic tissue of a leukemic-susceptible mouse was replaced by new tissue from the F_1 host, the leukemia that arose would grow in other F_1 mice but in neither of the ancestral stocks, thus conclusively demonstrating that the nature of leukemia is dependent upon the peculiar genetic constitution of the cells of the body. Trentin and Gardner[72] have performed a similar experiment with the induction of testicular tumors in mice by estrogenic hormone. Thus, the F_1 of a cross between a testicular tumor-susceptible strain (the A) and a testicular tumor-resistant strain (the C3H) was inoculated with normal testicular tissue from both ancestral stocks and then exposed continuously to a pellet of diethylstilbestrol. Only the testicular graft of a mouse of the susceptible A strain developed a testicular tumor (of Leydig cell origin). Therefore, the target organ and not the environment that the host can provide for the graft is the determining factor in the origin of testicular tumors induced by estrogenic hormone, and this end organ is shown to be determined by gene action. Finally, Gardner has performed a similar experiment in the induction of adenocarcinoma of the cervix in mice by grafting normal uteri from mice of two strains that differed in their responses to the same induction chemical-estrogenic hormone.

The application of the use of the F_1 individual to biological research appears to be limitless. A caution, however, should be expressed by not stopping the experiment in the F_1. It is very easy to conclude that gene action is responsible for different physiological characteristics in different inbred mice. Also, the appearance of a character in the F_1 seems to be evidence of dominant inheritance. Its failure to appear from the F_1 into the two ancestral stocks also indicates the mosaic nature of the F_1, as would be expected by complimentary genes such as were clearly demonstrated in the grafting of tumors that arose in F_1 mice of a cross between mice of the A and the D stocks. As geneticists, we should insist on testing for further evidence of genetic or gene influence. This evidence is not too difficult to obtain; the ratio of susceptibility to resistance in the F_2 should be mathematically consistent with the ratio obtained in the backcross to the recessive parent. When multiple genes are involved, the range of variability should be greater in the F_3 than it is in the F_2. Perhaps we expect too much.

Extensive advances have been made on the investigation of the so-called "histocompatibility" genes in relation to transplanted tumors in mice.

hese have been made possible by the study of linkage relations and the pplication of the component method for the determination of serologic haracteristics. The problem of linkage has been aided by the development isogenic strains of mice that differ, at least theoretically, only in 1 histo-mpatible gene. Isogenic strains of mice have been developed by alter-ating a system of F_1 and F_2 production with a backcross generation for any generations. Snell[61] found that 3 of the genes involved in the trans-lantation of tumors were linked with well-known morphologic or color genes s follows: (1) H_2 linked with fused on the ninth chromosome, (2) H_1 linked ith albinism on the first chromosome, and (3) H_3 linked with agouti on the fth chromosome. It appears, at present, that there may be a number of losely linked genes, at least 1 of which exists in 3 allelic series. Snell esti-ates at least 11 possible phenocopies involving the "H_2 locus." It is also ertain that, beyond these 3, there are other loci involved in the transmission f susceptibility to the transplanted tumor. Snell estimates a minimum of 2. It is also clear that the different loci have differential effects on trans-lantation. The H_2 locus is a "strong" locus, while the H_1 and H_3 have eaker influences upon susceptibility to transplantable tumors.

Recently it has been disclosed that the H_2 locus also has an effect upon the uccessful grafting of skin grafts. The histocompatible genes apparently ave effects, not only upon the growth of the transplanted tumor, but also pon the survival of normal tissue grafts. This finding further delineates the ifference between the transplantable tumor and the spontaneous. The echanism involved in the origin of the spontaneous tumor is not the same as he mechanism involved in transplantation. Perhaps it is better to consider he transplantation of tumors as a form of tissue culture or tissue survival in he foreign soil, whereas the spontaneous tumor originates from pre-existing ormal tissue of the same host.

Some old unpublished data of Strong would be of significance in the discus-ion of the differences between the transplantable and the spontaneous tumor. t was shown that a tumor that arose in a mouse of the D strain would grow rogressively in the presence of 2 genes (a 9:7 ratio in the F_2 and a 1:3 in the ackcross to the recessive parent, the A strain). The tumor, which grew n a mouse of the backcross, was then surgically removed and the mouse was bred to mice of the A strain. This process of selecting out from the backcross a mouse with the gene or genes for the growth of the transplant, the removal of the tumor, and further backcrossing to the A strain was continued for many generations. Thus, theoretically, there was obtained a mouse with a very large complement of the genes from the A strain, but with only 1 or 2 genes from the D strain, which were responsible for the growth of the transplanted tumor. Finally, this new strain, derived as outlined above, was inbred by brother-sister matings. Mice of this strain never developed spontaneous tumors, in spite of the fact that they definitely possessed the gene responsible for the growth of the original transplantable tumor. Perhaps a repetition of this experiment, using the greatly improved inbred strains of mice now in existence, may lead to further significant observations.

Aside from the differences that exist between the spontaneous and the transplantable tumors, the work of Heston clearly indicates the effect o specific genes upon the occurrence of spontaneous and chemically induced lung tumors in mice. The nature of these genes and their relation to the histocompatible genes involved in the grafting of transplantable tumors has not been investigated sufficiently to warrant a definite conclusion.

The application of the component method has disclosed that a single locus apparently has a multiplicity of serologic reactions. These specific reactions differ considerably in mice of the various inbred strains. To a geneticist this is a disturbing discovery. Perhaps conclusions are premature at this time. That a single gene may have a multiplicity of effects is an old theory that was altered by the classic investigations of Beadle and Tatum[8] in *Neurospora* and of Sonneborn[59-61] in *Paramecium*. Beadle and Tatum[8, 9] both maintain that a single gene does only one thing in biochemical reactions, and Sonneborn maintains that there is a 1:1 ratio existing between the genes and the killer factor (kappa) in the cytoplasm of *Paramecium*, even though kappa may be due to an invasion of a particle, as indicated by Preer.[51] Nor does the concept of pseudoallelism aid much, perhaps merely indicating that the original conclusion of multiple alleles was based upon insufficient data and that, had the investigator accumulated more data, the two genes might have been considered to be merely closely linked rather than as multiple alleles.

Some of the confusion existing in the interpretation of the component or serologic method of gene-complex identification may rest in the failure to recognize that, since many of the inbred strains of mice now being used had a common origin, they are mostly related to each other genetically. Therefore, by common descent, they must possess many genes in common. Being in the homozygous state, this cannot be indicated by conventional genetic tests of hybridization and recombination. In some cases, however, serologic tests are sufficient to distinguish between a heterozygous and a homozygous individual. Thus, the multiplicity of serologic reactions of a histocompatible locus eventually may be shown to be dependent, not upon a single locus, but upon many.

Several cases of crossing over between serologic component genes or loci already have been reported by Amos *et al.*[2] and by Allen.[1]

I cannot leave the transplantable tumor without emphasizing the ease by which its successful growth and survival can be influenced by experimental means. There are at least eleven ways by which tumor-host relationships can be altered, thus permitting tumors derived from one strain of experimental animals to grow progressively when transplanted into individuals of other strains, or even species. These methods of altering tumor-host relationships are being increased rapidly. At present they are as follows:

(1) X-raying mice before the transplantation of the tumor;[49] (2) X-raying the tumor tissue *in vitro*;[52] (3) mixing a tumor with cotton or wool fiber before transplantation;[34] (4) growing a tumor in the anterior chamber of the eye or in the brain;[24, 55, 56] (5) growing a tumor in an F_1 or backcross to the recessive-condition mouse[4, 27]—this observation is based partly on the fact that sus-

eptibility to transplanted tumors is dominant in crossing tests; (6) aging of umors through numerous transplant generations, permitting successive hanges to take place, that is, adapted changes that take place spontaneusly;[63, 64] (7) treatment of the host by estrogenic hormones before the injecion of the tumor;[19, 20] (8) treatment of the host by tumor or normal tissue xtracts before the inoculation of the tumor;[35, 57] (9) growth of tumors in the ewborn;[25, 42, 43] (10) passage of a tumor through the allantoic membrane of he chick;[47] and (11) conversion of a solid tumor into the ascites form.[22, 26, 39]

There appear to be different mechanisms involved in these different adapive procedures. Some methods (X ray) appear to alter the host mechanism s well as the tumor *in vitro*, whereas other procedures (4, 5, and 6) appear to bring about changes in the tumor cells or in tumor cell relationships. The mechanism involved in some of these adapted changes may indeed be quite complex.

A new tool for investigation of the cancer cell (that of the ascites tumor) has been developed recently by many scientists. The situation has been outlined by T. S. Hauschka of Buffalo, N. Y. His comments are as follows:

PRESENT STATUS OF THE RELATIONSHIP BETWEEN CHROMOSOME CONSTITUTION AND TRANSPLANTATION SPECIFICITY OF TUMORS

A cytological and genetic survey of 16 transplantable mouse tumors, most of them in the favorable ascites form, has shown an apparently constant correlation between modal chromosome number and host specificity.[29, 30] All of the near-diploid tumors examined consistently regressed in mice of foreign strains, while growths in the heteroploid category invariably gave lethal takes in from one to all seven foreign genotypes tested. This trend appears to be quite independent of the tumor's histological and genetic origin, method of induction, duration of serial transfer, virulence, and growth rate.

There is no conflict between the cytological and genetic data and the validity of transplantation genetics, as established through the classic experiments of Tyzzer,[73] Little,[43] and Strong,[62-64, 66] the serologic work of Gorer,[21] and the elaboration of histocompatibility loci and linkages by Snell.[58] On the contrary, some phenomena that have been puzzling geneticists can now be explained through known chromosomal mechanisms. Strong was the first to show that, during serial passage, a tumor may lose not just one but several isoantigens as a bloc, thereby finally becoming nonspecific. Chance coincidence of so many different point mutations is very unlikely. Changes in the opposite direction, that is to say, increases in specific transplantation requirements, have been very rare. These two observations are in accord with the upward shifts in chromosome numbers seen in various ascites tumors by Hauschka,[28] Levan,[44] Klein,[39] Sachs,[54] and others; the numerical changes have proceeded either slowly or suddenly from diploid to heteroploid—never in the opposite direction.

Addition of a single chromosome to a balanced diploid set of genes may affect the cellular antigens as drastically as doubling of the entire set, followed by subtraction of a few chromosomes. The latter is probably frequent, because modal chromosomes numbers between 70 and 78 are the most common among the polyploid mouse tumors, all of which are nonspecific.

The most essential feature of heteroploidy, in so far as it affects gene-controlled antigenicity of the cell, is genic imbalance. Neither point mutations in an H-2 locus nor physical loss of a chromosome carrying important H factors need be involved in bringing about antigenic surface changes. Since the molecular configurations of the lipoprotein cell surface probably are controlled by a variety of genes—antigenic ones as well as others—any change in the total dosage relationship of loci to one another might displace or alter molecules with specific antigenic function located in the cell surface. It is through the cell surface that the host recognizes or rejects the graft.

Cytological alterations that make some of the cells in a neoplastic population less ant genic would make these same cells more resistant to isoantibody produced by geneticall refractory hosts. Heteroploid cells thereby attain positive selection value. Usin immunoselection as an experimental tool in the weeding out of less antigenic cell type from a mixed neoplastic cell population has provided additional support for the inter relationship between heteroploidy and nonspecificity or reduced specificity. In two suc experiments,[31, 36] polyploid cells were selected across an immune barrier.

The most crucial documentation of decreased antigenicity as a consequence of aberran chromosome constitutions has been provided through the hemagglutination tests of Amos on three pairs of comparable diploid and heteroploid ascites tumors, the latter representin sublines of the former. In every one of these three comparisons the polyploid cells wer far weaker absorbing antigens than the corresponding diploid population.

The early observations of Strong,[63] Cloudman,[13] and others on stepwise losses of histo compatibility genes have been interpreted as indicating somatic mutations, and there i absolutely no disagreement between this interpretation and the findings of Hauschka,[2 Levan,[45] and Amos.[3] Loss of specificity due to heteroploidy concerns only the final step from a difference involving one or several genes to the total absence of an antigenic barrier between graft and formerly resistant host. This final step, too, could of course come about through point mutations. However, the evidence favors a wide variety of gross chromosome number changes as the most frequent cause of genetic indifference in tumors.

T. S. HAUSCHKA

The introduction of a discussion of the heteroploid transplanted tumor has been rather extensive. This was necessarily so, since the development and even the continuation of the genetic concept of cancer is dependent upon the evaluation of the data obtained primarily with the ascites tumors. Hauschka has clearly indicated his viewpoint. I shall return to these observations again. At present, perhaps it will be wise to clear up what appears to be, from the examination of the literature, a discrepancy of interpretation. Hauschka quotes me to the effect that "during serial passage, a tumor may lose not just one but several isoantigens as a bloc, thereby finally becoming nonspecific." Are we justified, however, in concluding that "blocs of genes" are or were lost or altered simultaneously? The original data, instead of making this conclusion obligatory, never indicated that such was the case. Where multiple genes are involved, the differences between ratios of susceptible to resistant transplantable tumors are very slight. In order to distinguish between the number of genes responsible for the survival of the tumor, one must have a considerable colony of mice—more than ever were available to me between 1922 and 1926, when the experiment was done. Again, the detection of altered or derived transplants was based upon alterations in growth rates of the tumors. With the number of animals then available, considerable time had elapsed before proof of a significantly increased growth rate of tumors could be obtained. When the altered transplant with an increased growth rate was then tested, it appeared as though a whole bloc of susceptibility genes had been detected. However, this observation should not be accepted as the entire story, but rather as a stage in a progressive breakdown of tissue specificity that determines tumor transplantation. The final conclusion concerning the extent of these progressive and successive sudden changes can come only from the repetition of the experiment—starting not with a transplanted tumor that has been carried for several months or even years, but with the spontaneous tumor, and testing out the ios of successive transplants in adequate members of F_2 or backcross to e recessive-condition mice. Some new evidence of Klein,[38, 39] where four five genes identified with tumor susceptibility apparently are rendered mpotent" or are inactivated by growing the tumor in an F_1 individual, eds further consideration. However, the final conclusion should rest on whether the altered change is permanent or only of a temporary ture. Here we need cytological evidence obtained simultaneously with e genetic evidence.

The concept of the so-called histocompatible gene has been greatly vanced by the recent work of Amos, working in Gorer's laboratory at uy's Hospital, London and in Hauschka's laboratory at the Roswell Park emorial Institute, Buffalo, N. Y. Amos comments as follows:

PRELIMINARY STUDIES ON THE ANTIGENIC RELATIONSHIP BETWEEN COMPATIBLE TUMORS OF DIFFERENT CHROMOSOME NUMBER

The purpose of the experiments was to determine whether there was an alteration in tigenic activity referable to changes in chromosome number away from the diploid. he material studied by Hauschka and Levan provided evidence that tumors with a diploid near-diploid population were strain specific, whereas aneuploid tumors would grow proessively in at least one foreign strain.

The three comparable tumor pairs available for study were (1) the diploid C3H lymhoma, 6C3HED, and a derived polyploid subline adapted to grow in DBA/2 mice; (2) e diploid A No. 2 lymphoma and the polyploid A No. 1 lymphoma; and (3) the near-ploid A-strain carcinoma TA-3 with its derived polyploid subline TA-32.

When ascites cells from these tumors were used to absorb hemagglutinating antibodies om sera made against a variety of unrelated tumors of known H-2 composition, the llowing differences were found (in the case of the A lymphomas a similar result was btained by Hoecker,[33] using an I.R. anti D, K serum):

(1) Diploid A lymphoma No. 2 would absorb out all the antibodies against A-strain ntigens C, D, E, F, and K. Near tetraploid A No. 1 would not absorb antibodies against , E, and K. There was variable activity against anti D and anti F, best shown with eak antisera.

(2) Diploid 6 C3HED would absorb antibodies against the H-2 k antigens C, K, and E, ut somewhat less effectively than would normal C3H liver.

Polyploid DBA/2-adapted 6 C3HED tumor tissue would not remove these antibodies. hen transferred to C3H mice, the diploid cells present (6 per cent) in the ascites fluid apidly outgrew the tetraploid. This change in population was associated with ability to bsorb anti K.

(3) Diploid TA-3 would absorb out anti D, E, F, and K (C not tested). Polyploid TA32 ould not absorb out C, D, or E, but appeared to have more affinity for anti F. Antiodies were then made against two of these pairs.

The antibody prepared in C57BL (H-2 B) against 6C3HED was a high titer and appeared be a typical anti H-2 K serum. It was absorbed by normal C3H liver. The antibody gainst the DBA/2-adapted form was a weak anti D. It was not absorbed by C3H liver, ut was easily removed with DBA/2 liver. The antibody against L No. 2 prepared in 57BL mice was a high titer antibody containing both anti D and anti K. It was readily bsorbed by L No. 2 cells, but not by L No. 1. C57BL anti L No. 1 was a weak anti , readily absorbed by L No. 2 and L No. 1 cells. (The weak anti D against polyploid C3HED is most probably due to contamination, since the tumor was taken after being aintained for a long period in DBA/2 mice and contained an unknown number of DBA/2 ells.)

The weak anti D produced against L No. 1 cells is probably a genuine phenomenon, since ransfers are made at frequent intervals (the tumor being maintained in the A strain), and contamination with A-strain cells should have resulted in the production of both an K and anti D. No significant amount of anti K could be detected. It is interesting th this tumor will grow in DBA/2 animals having the constitution H-2 d, but not in mice the C3H or other strains.

From absorptions made against the C57BL anti 6C3HED serum with 6C3HED cel it appears that this tumor was more active against this serum than against a comparab titer serum against a C3H mammary tumor. Some reduction in titer for C3H cells w obtained after absorption with the tetraploid form, although the latter had no affinity f an antimammary tumor serum. Comparisons made at the same time with liver, kidne and muscle from C3H mice indicated that these tissues were slightly more active at absor ing anti-mammary tumor serum than the antibody against the 6C3HED lymphoma.

Antibodies against the diploid tumors will protect against these tumors in foreign strai and less effectively in the susceptible strains. Comparison with antibodies against th aneuploid tumors will be made.

Elsewhere in this monograph, Strong has discussed changes in Mendelian ratios foun on repeated transfer of a tumor. He has pointed out that when the transplanted tumo begins to grow in all F_2 mice, it then grows progressively in mice of foreign strains. Som of the stages of genetic simplification may be reversible, as has been shown by Hauschka, by passage through the F_1. Gorer and Amos[23] encountered a similar situation whe inoculating an F_2 that had previously been immunized with blood from the C57BL leukos E.L.4. To date, however, no such reversal has been demonstrated in the final stage, tha is, a transformation of a completely nonspecific tumor to the original specific form.

Snell[61] has shown that the most effective single factor or gene-determining transplantation is linked with the ninth chromosome group, and Gorer[21, 22] has found that this, th H-2 locus, can be identified serologically. Recently, what was at first thought to be a singl locus has been shown to be a complex containing at least five components closely linke but spatially distinct, thus permitting crossing over between at least some members of th group. Most of the serologic work has been done with strain-specific tumors which together with some normal tissues, notably liver, have been found to be rich in H-2 antigens

Hauschka and Levan,[29] in an analysis of about sixteen tumors, have established a correlation between strain specificity and chromosome constitution. Tumors having a diploi or near-diploid complement are strictly strain specific, and those having even a moderat degree of chromosomal imbalance violate some or all strain barriers. Hauschka postulate that there was probably an immunological basis for this distinction, the aneuploid tumor having reduced antigenicity. Hoecker[33] believed that an increased chromosomal complement would more probably be associated with an increase in antigen, but later showe that the aneuploid A-strain lymphoma No. 1 was less antigenic than the diploid A lymphoma No. 2.

Immunological studies now have been made on a large number of tumors and are bein continued as new tumors become available. The most intensive study has been performe with three pairs of closely related tumors: the diploid A-strain carcinoma TA-3 and it derived polyploid variant TA-32; the diploid A lymphoma No. 2 and the near-tetraploi lymphoma No. 1, these two tumors being induced in litter mates; and the C3H lymphosarcoma 6C3HED and its polyploid variant-adapted DBA/2. These data have been give in more detail previously.

The presence of antigens has been tested in two ways: (1) by absorption of antibodie by a packed suspension of ascites tumor cells, and (2) by eliciting an antibody respons against the tumor cells by a series of injections into mice of foreign strains. The concentration of antibody in both cases was usually estimated by hemagglutinin titration.

By these methods the polyploid tumors have all been shown to be markedly less antigeni than the corresponding diploid or near-diploid cells. By absorption tests it has been foun that the polyploid tumors are deficient in some of the antigens present in the diploid for or in normal tissue; the antibodies produced against the polyploid tumors were of ver low titer. They appeared also to be directed largely against the few contaminating hos cells in the inocula. By a more intensive and prolonged course of immunization, antibod is eventually produced. Evidently the antigen is still represented, but in reduced amoun It was suggested that the antigen could be present within the cell, but fails to reach th face of the polyploids. Attempts to demonstrate this by grinding up the cells before orbing did not indicate the liberation of the antigens from the interior of the cell.

mmunological methods have been used in the study of a number of other tumors, both loid and aneuploid, some recently derived and some long established. All have followed pattern of the three pairs described above, with one exception. This was the C3H cinoma MC1M. An additional group of twelve spontaneous neoplasms have been ted for their absorptive capacity and have proved to be strongly antigenic. A total of teen strain-specific tumors has been examined by various immunological means. All ve been strongly antigenic.

The exceptional tumor, MC1M, which has a chromosomal mode around 70, will grow a proportion of mice of a number of foreign strains. It is, however, strongly antigenic. is an unusual tumor in at least two other characteristics: (1) it forms large quantities a highly viscous substance when grown as an ascites tumor, and (2) its most frequent thod of division is by endoreduplication.

The general interpretation of the results so far obtained is clear. In their earlier stages e great majority of tumors are (1) strain specific, (2) have a high Mendelian ratio in netic experiments, and (3) are highly antigenic. With repeated passage there is a pro-essive simplification in genetic requirements, the end products of the H-2 locus remain ore or less constant, although at times one component may be more in evidence than hers. The tumors are diploid or near diploid in their chromosomal constitution and main strain specific. That some of the genetic complexity has been retained can be own in certain cases by indirect methods cited above. In the final change, (1) the endelian ratio drops to less than one, (2) the tumor can establish itself in mice of formerly sistant strains, (3) chromosomal imbalance becomes apparent, and (4) there is an altera-on in the H-2 antigens.

These processes can be explained if it is assumed that the earlier changes are a response a continuing "parasitic" environment in which complex metabolic processes constitute competitive disadvantage. Those cells with less complex end products to manufacture nd with a more rapid growth rate would replace the more complex cells. This would be ssociated with a decrease in antigenicity (the antigens being the end products of metab-ism) and an increase in growth rate. Both of these phenomena are consistently encoun-ered. The *final* change is that involving the H-2 locus, which apparently has been unaf-cted in earlier changes. The correlation between chromosomal imbalance and the altera-ons at the H-2 locus and the apparent irreversibility (and irregularity of occurrence) of is change suggests that it is mutational—a mutation at the H-2 locus resulting in a com-lete upset in cell economy.

Inferences and Conclusions

For the tumors TA-3 and TA-32, the TA-32 polyploid tumor is very much less antigenic, s judged by absorption, against all the sera tested. The diploid tumor appears to behave ke A liver or anti A-strain tumors previously tested.

The A No. 2 diploid lymphoma also appears to contain the normal A-strain antibodies, oth in absorption, antibody production, and protection experiments. The polyploid A To. 1 appears to retain a little of the D (and possibly F) antigen, but no K or E.

The diploid 6C3HED retains the H-2 antigens K and E, but possibly in a slightly ltered form. The polyploid form has apparently no normal K or E, but may contain a ittle of the altered K present in the host tumor.

The experiments showing that some well-established tumors can be made to increase the pparent number of factors required for transplantation may mean that the ability to form ntigens other than H-2 may be "waived" in serial passage without alteration in strain pecificity. This may be associated with an increase in growth rate of the tumor, and is daptive (it may be a parasitic type of phenomenon in which the necessity for some meta-olic processes is not required in a favorable environment). The change in H-2 is possibly different process involving complete reorganization of the economy of the cell, and may e associated with morphologic and growth-rate changes; in which case the H-2 locus could e considered to elaborate some essential process for normal cellular economy, which is, ncidentally, strongly antigenic.

Alternatively, in the alteration from diploid to tetraploid, an alteration in surface co figuration restricts the H-2 antigens either to the deeper layers of the cell membrane or the interior of the cell. This hypothesis may be checked by absorptions made with c fragments.

D. B. Am

It was unfortunate that the concept of changes in tissue specificities transplantable tumors was brought about by “losses of histocompatibili genes.” First, if a mutation is involved in the origin of cancer, then canc should be regarded as a deviation of the normal cellular make-up and thu would be histoincompatible from its tissue of origin even from its ver inception. Perhaps this is not significant, since the normal allele of a hist incompatible gene could be considered as histocompatible. Second, howeve a more significant criticism of the use of the term “losses of histocompatib genes” rests on the fact that the concept continues the old idea of th “presence and absence” theory of William Bateson, which has been abandoned in genetics for many years. Perhaps it would be wiser to refer t these genetic changes, not as losses, but as alterations or merely as change in gene structure or organization. The term “replacements” would not b too ambiguous.

One comes face to face with this problem of terminology in considerin the polyploid tumors in relation to the older work on the genetics of th transplantable tumor. Apparently there is a continuous series involved i the progressive changes taking place in the transplantable tumor. Certainl the work of Amos verifies this concept that the polyploid tumors are les antigenic than their diploid counterparts. On the surface it looks as thoug the breakdown of tissue specificity is due to losses of histocompatible gene that act as antigens, whereas the polyploids appear to have “gained” something (one or more chromosomes) that still gives less antigenicity.

Two facts should be kept in mind when dealing with polyploidy in reference to cancer. First, polyploidy should be considered in the way tha Blakeslee[11a] and others have done in the Jimson weed, where all sorts o chromosome aberrations have been observed without the presence of cancer In *Datura* there appear to be many combinations of chromosomes, some o which are compatible with the continued existence of the plant, wherea others are apparently incompatible. Thus, balanced and imbalance chromosome components have been discussed, and cancer is not involved Similar concepts are now being accepted in the field of cancer research, a evidenced by references to such phenomena as balanced and imbalance hormonal states.

Again the observations of nondisjunction should be introduced. It ha been known that three recessive genes, obtained by the addition of a singl chromosome, would in certain cases produce the dominant state. It is als probably true that the chromosome is nothing more than the sum total c the genes it contains. Such phenomena as position effects of spatiall related genes and the relation of euchromatin to heterochromatin should b considered here, although it must suffice here to point out merely that th addition of extra chromosome material would of necessity alter the spati relationships between genes.

n heteroploidy one is not only duplicating a whole chromosome that can seen through the microscope; one is also reduplicating genes that can be icated only by suitable genetic tests. Thus, three altered or changed nes may revert to the wild type, and four or five may even exceed the called normal. Perhaps in cancer one should not expect to find the same netic phenomena that occur in so-called normal tissues.

It is therefore probably true that the genetic changes in the potentialities transplantable tumors are the result of a single sequence of events. The ocess leading to so-called less tissue specificity, as determined by the lity of tumors to grow in a variety of animals, is progressive but reversible, least to a certain extent. Whether cancer is the end result or merely a ge of intermediate significance cannot be visualized. If intermediate ly, what is the end? One could speculate that the organism has slowly olved from a system of complex organization. Is cancer a reverse process which the organism has lost the controlling influence of all or some of its rts? If this is so, then why do some, but not all persons, develop cancer? hat is the real significance of cancer in the biological series?

A great stimulus for the continued interest in the mutation concept in erence to the origin of cancer was provided by the observation that ethylcholanthrene (one of the most powerful carcinogens) was a mutagen least in mice[67, 68] and in *Neurospora.*[71] The spontaneous mutation rate my[68] colony of controlled inbred mice has been 9 mutations in 232,000 ce, or 1 mutation in 23,777 mice (mutation rate, 3.9 per 100,000). In the scendants of mice injected with methylcholanthrene, 12 mutations have curred in 8400 mice, or 1 mutation to 700 mice (mutation rate, 142.8 per 0,000). The "t" value between these two ratios is 20.4. Thus, in this d in many other of my experiments methylcholanthrene was a mutagen. atum has concluded that methylcholanthrene is a mild mutagen in *Neuro-ora*, and Tatum and Barrett have reported an increased mutation rate hen the methylcholanthrene derivative had been made slightly more water luble, based upon the idea of aiding the carcinogen's entry into the cell.

The production of such a large series of mutations in my series of mice bjected to methylcholanthrene for many generations, and the cursory porting of negative results by other investigators following one injection the carcinogen, led to the suggestion that perhaps my series was due to genic instability." Genic instability, or a very high spontaneous mutation te, has been reported by Demerec[16] in *Drosophila*, and by other investi-ators. This genic-instability concept was tested in the mice subjected to ethylcholanthrene and found to be untenable.

The new data further verify the conclusion that "there have been no eculiar genetic phenomena (such as genic instability) other than clear-cut oint mutations in the origin of 15 mutations following the subcutaneous jection of methylcholanthrene." These mutations are associated with ethylcholanthrene or one of its metabolites, and must therefore be consid-ed as chemically induced.

Four series of mice are being continued in the laboratory. These are as llows: (1) the untreated controls, consisting of mice of the original Strong inbred strains, the A, C3H, CBA, N, etc; (2) the Br's, or NHO descen which has now been injected with methylcholanthrene at 60 days of li for more than 40 inbred generations; (3) the separation off of descents fro mice that have developed mutations following the injection of methy cholanthrene into parental lines of mice for many generations and have bee freed from further injection of methylcholanthrene; and (4) hybridizati crosses necessary for the genetic analysis of mutants that have occurred series 2.

Two new spontaneous mutations have occurred in series 1. These hav been (1) pintail in a mouse of the C3H strain, and (2) a mutation from *a* A or A^1 in the original untreated NHO descent. A mutation in the C3 strain has occurred in one of the original Strong inbred strains. The origin inbred strains serve as the background control group for the investigatio of spontaneous mutations under the existing laboratory conditions of th Springville laboratory. This colony has been continued intact for more tha 35 years. The second mutation of *a* to A or A^1 occurred in the second seri of controls (the original NHO descent), which has never been injected wit methylcholanthrene, but which gave rise to the various sublines (Br, pB Brpb) that have been injected with methylcholanthrene for many generatio (that is, that gave rise to series 2 and 3 of this report). This spontaneou mutation (*a* to A or A^1) is of considerable interest or significance, since it the first reverse mutation that has ever occurred in a population of more tha 500,000 mice over a period of 35 years.

There have been no further mutations in series 3, where a descent has bee established from a mutation that has occurred in a mouse whose ancestr had been injected with methylcholanthrene for many generations and whic was freed from further injection of methylcholanthrene as soon as th mutation had been obtained. The evidence is thus conclusive that gen instability was not involved in the origin of these chemically induce mutations.

One descent consisting of 3 color mutations of brown to black, self to pie bald, and color to chinchilla has been continued free of methylcholanthren since its appearance 20 generations ago. No mutations have appeared more than 15,000 mice. The Br subline of the NHO descent has been sep rated from methylcholanthrene treatment for more than 25 generations an no mutations have occurred. Two descents have been separated off fro the pBr subline of the NHO—one of these in the F_{17} following treatment wit methylcholanthrene for 14 generations, the other in the F_{20} following trea ment with methylcholanthrene for 17 generations. The original pBr descen and the 2 derived sublines (Prunt and 2-Prunt) have been separated off fro methylcholanthrene for many generations and have now reached F There have been no color or morphologic mutations detected in these descents during this time. This evidence is highly significant, since th original pBr descent exposed to methylcholanthrene for several generatio gave rise to 3 color mutations, one being a reverse mutation from p to This dark-eyed mutant, obtained in a mouse whose ancestry had been injecte with methylcholanthrene, was continued for 14 generations in the heter

gous condition by mating a dark-eyed mutant (pP) to a pink-eyed sib
p). During these 14 generations of inbreeding, the descent produced 16,251
ce, 8115 of which were mutant dark eyes and 8136 were pink eyes. A
1 ratio for this number of mice would be 8125.5 to 8125.5. There were
new mutations in this untreated descent. This mutation from p to P
of interest since it is a reverse mutation, that is, from the recessive condi-
n back to the wild-type dominant. Similar mutations in *Drosophila* are
aracteristic of genic instability (see Riley,[53] page 239).

No evidence of genic instability, however, could be obtained in this reverse
utation, since normal segregation was occurring in its descent and no other
utations at other loci were obtained.

The inheritance of polydactylism has shown some very interesting bio-
gical facts. Polydactylism occurred upon 4 separate occasions in mice
hose ancestors had been injected with methylcholanthrene for many gen-
ations. Since the condition shows at least partial dominance in inherit-
ce, although low penetrance, it may be that 4 mutations of independent
igin were obtained. The condition is sensitive to maternal age, and new
idence demonstrates that the effect of maternal age is cumulative over a
umber of generations. In the F_1 generation of a cross between a male
ouse showing polydactylia and female mice of the C57 strain, the highest
gree of penetrance was obtained when the mothers were more than 200 days
d at the time of conception. This percentage in mothers of 201 to 300
ys of age was 3.38, compared to 2.08 per cent when the mothers were
tween 100 to 200 days, and 2.11 per cent when the mothers were between
00 to 400 days. The number of F_1 young upon which these percentages
ere based were 1034 normal to 22 polydactylias; 1083 normal to 83
lydactylias; and 372 normal to 8 polydactylias. Four selected sublines
e being continued, based upon the age of the mother at the time of con-
ption. These are (1) less than 100 days, (2) between 101 to 200 days,
) 201 to 300 days, and (4) 301 to 400 days. A maximum penetrance of
5 per cent of polydactylous offspring has been obtained in the F_{12} generation
the early-age descent. Penetrances in the other 3 selected sublines are
gnificantly lower than this maximal penetrance. In this early-maternal-
ge polydactylous descent, several other mutant or biological variants have
ccurred. These are syndactylia, brachydactylia, open eyelid or olive,
uxate appendages, flexed tail, umbilical hernia, and anemia. In addition,
cases of flexed tail and several cases of hydrocephalus and "loop tail" have
een obtained. The biological relationships between these various mutants,
r biological variants, are being investigated. At present it appears that
uxate is not genetically identical with polydactylia, but may have a modi-
ying effect. So far, 71 mice showing luxate have been obtained by crossing
mice, both of which had polydactylia on both hind feet. All luxates are
escendants of a single male mouse showing a triple hallux. All luxate can
e separated from polydactylia, since several mice have occurred with
olydactylia on 3 feet, and even with polydactylia on all 4 feet.

The production of such a large series of morphologic characteristics in one
escent whose ancestry had been injected with methylcholanthrene for many

generations continues, therefore, an interest in genic instability. Howeve complicating factors have already arisen and must be analyzed further. It of interest that all of these morphologic characteristics have occurred only i the early-maternal-age descent. If genic instability is involved with thes many morphologic characteristics and mutants, then genic instability ma be correlated with an early-maternal-age descent. If this is so, then perha we are dealing with the fundamental biological mechanism of gerontolog It is true, however, that the late-maternal-age sublines do not produce a good breeders as the early-litter descent, and several of the later-materna age-descent sublines have already died out. Consequently it become imperative to study divergence of morphologic mutants and variants i separate selected sublines in the intermediate classes of parental age, wher differences are smaller and therefore require larger numbers, but will b conducive to the production of significant numbers within a reasonable time

Injecting methylcholanthrene into a series of mice over many generation and then freeing the descent from further exposure to this chemical continue to produce a great variety of biological effects besides cancer, some of whic are clearly genetic.

The reports of negative observations on mutagenesis with methylcholanthrene by several investigators have not been too convincing. For example, one experiment was stopped in the F_2, following the injection of methylcholanthrene into the abdomen. It is recognized that no induced recessiv mutations could be expected until the F_3.

Again, the placing of methylcholanthrene into the diet upon which *Drosophila* have been kept is perhaps not wise, since there is no evidence tha methylcholanthrene could pass through the gut wall of that species. Loren and Stewart[46] did report the induction of tumors in mice that could b explained by a passage of the carcinogen through the gut wall. Are w justified in concluding that the absorption of the material in the Diptera i the same as absorption in Mammalia?

Other complications involved in the testing of certain carcinogens such a methylcholanthrene for mutagenic properties are (1) the great insolubilit of the chemical and (2) its peculiar chemical reactions. Part of this difficult can be overcome by making the carcinogen more water soluble, as was don by E. L. Tatum (oral communication). The second difficulty can perhap be lessened by permitting a longer time of exposure before the mutageni property is measured. In the experiment I performed[66] this criticism wa anticipated and partly met by subjecting the methylcholanthrene-treate mice to a rigid regime of selection toward resistance to all kinds of chemicall induced tumors. Germinal mutations did not arise until the animals ha lived long enough to develop late-appearing abdominal tumors, includin ovarian, thus proving that some component of the injected methylcholanthrene was getting into the ovary. Whether the injection of methy cholanthrene into mice for many generations before the appearance of th germinal mutations was a contributing factor can be conclusively demon strated only by repeating the experiment; so far, this has not been done.

Continuing the parallel between mutagenesis and carcinogenesis is som

at hampered by the observation that, in the descendants of mice receiving
thylcholanthrene over a number of generations, many nongenetic varia-
ns such as phenocopies have been obtained. For example, 7 variations
olving the tail in mice have been obtained in the descendants of mice
ected with methylcholanthrene. Three of these were genetic (pintail is a
minant, showing linkage with brown), while 4 have been proved non-
etic. Thus, the real role of methylcholanthrene in the origin of cancer
again become vague or uncertain. In order to make biological sense out
the vast array of information now available one may therefore be forced
assume that methylcholanthrene is not the true mutagen or carcinogen,
that in its metabolism more than one intermediate is formed and that
h one of these intermediates may be responsible for these different bio-
ical phenomena.

ne other series of experiments of genetic importance should now be con-
ered. This series, dealing with the nature of the adaptation of the trans-
nted tumor, stems from the work of Barrett and Deringer[4] of the National
ncer Institute, Public Health Service, Bethesda, Md. Barrett,[5, 6] and
er Barrett and Deringer,[4] have reported that the growth of the transplanted
nor in an F_1 obtained by crossing a mouse susceptible to a transplant to a
use of a resistant-strain (or recessive) parent could serve as a bridge by
ich the tumor successfully growing in the backcross would later grow suc-
sfully in increased numbers of backcross mice. This work has been veri-
d by Hauschka and has led to some fundamental concepts of the nature of
transplanted tumor. My series of experiments can now be reviewed.

One of my papers[69] deals with a series of experiments in which 1948 mice
re inoculated subcutaneously with a spindle cell neoplasm that arose
ginally in the forestomach of a mouse of the pBr descent. The mice
ed belonged to the pBr and N inbred strains and to the F_1 and F_2 genera-
ns of a cross between the pBr and N. Following 9 generations of growth
mice of the N strain, the tumor (N-adapted) was returned to the original
r mice for 13 generations and then into a new group of F_1's and F_2's.
e original tumor from the pBr strain gave a $15+:1-$ ratio in a group of
mice. This would indicate that progressive growth is dependent upon
her of 2 alternative genes. The N-adapted tumor gave a $9+:7-$ ratio
pected when 2 genes were simultaneously needed for progressive growth
the tumor. From a dependent tumor the transplant became more depend-
t through some change of a genic or factorial nature. The phenomena of
aptation and changes in the transplantation potentials of tumors are thus
dicated.

In a second paper,[70] dealing with the same problem of the adaptation of
e transplanted tumor, I have determined that the spindle cell neoplasm
ich arose originally in the forestomach of a mouse of the pBr strain will
t grow in mice of the F strain either directly from pBr mice or from mice
the F_1 generation of a cross between pBr- and F-strain mice. Thus the
rk of Barrett and Hauschka, with the work on other transplantable
mors, cannot be verified.

The spindle cell neoplasm, after having grown in mice of the N strain for

34 generations, was returned to mice of the pBr strain and given the symb "3N-adapted" tumor. Following residence in pBr mice for several trans generations to accustom it to its original hosts, it has been determined th the 3N-adapted tumor will grow in all mice of the F_2 generation of a cro between mice of the N and pBr strains. In other words, the 3N-adapt tumor is now nonspecific, showing no segregation of genetic determiners in cross between mice of the N and pBr strains.

The 3N-adapted tumor will not grow in mice of the F strain either when is obtained from mice of the pBr strain or from an F_1 individual derived fro a cross between F- and pBr-strain mice. It seems justified, therefore, conclude that changes in the adaptive mechanism involved in the transplant tion in mice of the pBr, the N, and the 2 hybrid generations derived from cross between these 2 strains is selective or specific. As far as can be dete mined, these changes do not involve the mechanism of growth in mice of t F_1 and F_2 generations derived between mice of the F and pBr strains. T investigation is suggestive of the fact that the adaptive changes taking pla in the mechanism of transplantation are not fortuitous or brought abo exclusively by chance alone. The host may be instrumental in determinin to a certain extent, these adaptive changes. This would mean that not of the characteristics of the tumor are independent of the host.

It has been maintained that the mutation concept in regard to cancer only of academic interest—that is, if a mutation (somatic) is involved in th origin of cancer, then nothing can be done to prevent the onset of cancer even to control it by therapeutic means. The only protection man wou have against radiation as the source of mutations would be heavy shields lead. This does not necessarily follow with the control by treatment, how ever. It is commonly accepted that the mechanism involved in the con tinued growth of cancer within the body is entirely separate from the mecha nism involved in its origin. Thus, environmental means could be devise for treatment, even though the cancer originated, in the first place, by somat mutation. The mutation concept is of more than academic interest, how ever, since any rational treatment of a disease should be based upon its tru biological nature. A biologist should not become discouraged in trying prevent the onset of cancer, even though a mutation may be involved in i origin. By investigating antimutagens (other than a shield of lead) may be able to prevent cancer and, at the same time, make some fundament contributions in the field of genetics.

If a somatic mutation is involved in the origin of cancer, and if there any valid comparison between mutagenesis and carcinogenesis, then furth contributions should be sought. There is space here to cite only tw possibilities:

(1) Cancer susceptibility is intimately associated with the process of t aging of the individual. All cancers and tumors, in both man and anima have a frequency distribution according to the age of the individual. F example, cancer of the skin increases in frequency with increasing ag Does the mutation rate increase with the age of the individual?

Dahlberg[15] has anticipated this idea and, in 1940, he maintained that " not been observed that mutations in cells of the gonads are more frequent lder than in younger persons." However, Blakeslee[11b] has demonstrated t the mutation rate in *Datura* is increased by the aging of the seeds. 2) It is becoming increasingly evident that cancer susceptibility and such cesses as longevity in Cladocera, Mongolian idiocy in man, and other racteristics, are associated, not only with the aging process of the individual, but also with the aging process of the parents (especially the mother). mutations occur more frequently in the offspring of mothers as mothers ome older? Correlations here would greatly strengthen the concept of a allelism between mutagenesis and carcinogenesis. Again Dahlberg[15] has d his viewpoint to distinguish between somatic mutation and vegetative roduction and, by so doing, he has outlined a program by which this second blem can be investigated. He states that "from this point of view a per- who has died of cancer at the age of 90 years must have a comparative k tendency to form tumors. So we should not expect his relatives to w a significantly higher frequency of cancer than other folks."

ahlberg[15] also reports (1) that persons who died of cancer but whose ents have not died of cancer lived 54.57 ± 0.29 years; (2) persons who d of cancer and whose parents died of cancer below the age of 60 lived 73 ± 0.91 years; while (3) persons who died of cancer and whose parents d of cancer above the age of 60 lived 57.06 ± 0.94. The difference ween 2 and 3 is 4.33 ± 1.30 years, which is more than 3 times the standard or. Dahlberg despairs of obtaining sufficient evidence bearing upon this cept, however. Under controlled conditions, similar studies could be formed on experimental animals.

t would be just as important to find out what happened to the younger d the older brothers and sisters of the cancer probands. Only after this ormation is made available can we distinguish between quantitative levels genetic transmission of cancer susceptibility and the aging process in the rents, especially the mother.

There are other fundamental biological problems that need investigation. r instance, we in Genetics have been so impressed with the dynamic luence of specific genes and gene combinations on development and characteristics of the individual that we have overlooked the medium cytoplasm d the environment in which the genes must function.

Summary

I have attempted to orient the problem of the genetic concept of the origin cancer, to indicate the contributions that have a bearing upon it, and to int out the limitations of the experimental approach. Only time will tell ether we are on the right track.

Investigators in cancer research have borrowed extensively from the ideas genetics. Some of these have been outlined here. At first this borrowing s meager. With the passing of time and the development of the many netic contributions now available, this borrowing has rapidly increased til at present the tempo is considerable. Not all of these contributions can be indicated. Many of them are appropriate and should be discuss before a complete genetic concept of cancer can be postulated. The t chief fields that may be of interest are (1) cytoplasmic inheritance and (mutations, particularly in microbiology, the bacteriophage, bacteria, a viruses. We cannot discuss cytoplasmic inheritance and formed cytoplasm components without introducing the topic of gene-cytoplasmic dependen or parallelisms. At least one fact must be kept in mind in concluding that cytoplasmic component may be involved in the formation of the cancer ce and this of course also applies to the gene or chromosome. Whatever t mechanism within the cell that makes it cancerous, it must be a self-perpet ating, fairly permanent component that can duplicate almost indefinitely specific effect upon the rest of the cancer cell as a unit by cell division. Sor of the transplantable tumors have retained an individuality for more tha forty years, through literally thousands of serial transfers.

The field of mutation is an ever-expanding one. We now hold sympos rather than publish single papers such as the pioneer one by Muller radiation-induced mutations in *Drosophila*. Here again only a few commen can be made, and these only so far as the cancer problem may be involve The oxygen effect on induced mutations is quite confusing. Does it have direct or an indirect effect? When this confusion has been cleared up it ma be time to apply the oxygen effect to the cancer problem, but it should emphasized that oxygen tension, especially reduced oxygen effect, and all biological ramifications has intrigued cancer investigators for more tha fifty years, particularly Clowes and Keltch[14] and, very recently, Urbach and many others.

The use of temperature gradients has had the reverse application. It h been shown that temperature changes influence the genetic phenomena crossing over and the somatic manifestation of gene action. Only recent have cancer investigators begun to use temperature for such purposes determining differences in cell populations of ascites tumors.

Both mutagenesis and carcinogenesis are variable quantities in differe species; it is perhaps wise to compare them in the same species. For th purpose the mouse is the species par excellence. The wealth of differe inbred strains that differ in their capacities to give rise to all degrees of su ceptibility and resistance to many types of tumors and cancers is attracti an ever larger group of investigators. Comparative studies have been ma possible by the reduction of the uncontrolled biological variability th existed originally in so-called commercial or market mice and which st exists in most species available for experimentation, and even in man.

There is one point that must be kept in mind in attempting to cont cancer through the investigation of mutation. Even if a mutation is involv in the origin of cancer, the tumor certainly continues itself through c division or mitosis and not by successive mutations. The best one wou hope to accomplish by the control of the mutation process would be to p vent the onset of cancer, never its control when once present, or its cu Pathology has taught that the origin of cancer is an entirely different proble

n its continued growth. It seems reasonable to expect, therefore, that
control or cure of cancer (at least the experimental type) should come
n investigations dealing with the control of cell division or the control of
mone imbalance that may be involved, by the control of metabolism of
cancer cell by such compounds as antimetabolites, or by the empirical
lication of atomic energy or selective toxic or poisonous compounds that
uld kill the cancer cell in preference to the surrounding normal tissue.
haps the trial-and-error method of chemotherapy may provide the answer
ontrol.

Many other concepts of genetics are now being introduced into cancer
earch. These will be carefully considered and accepted or rejected on
ir intrinsic merits. However, some of these concepts need not confuse
cancer literature, as for example, the idea of "genic drift" that is begin-
g to appear in the cancer literature. Perhaps this suggestion stems from
unexpected observations of unpredictable variations obtained by the use
extremely powerful chemical and biological agents on relatively large
nbers of animals over a period of time. Part of this variation is due to the
sconception that, by the use of inbred mice, one can obtain homozygous
ce. A strict brother-to-sister mating for many generations will not con-
ue highly inbred mice unless all collateral or side lines are eliminated and
y a very restricted parental line is continued. In each generation the
st looking or healthiest pair have been continued as parents, and these
y very well be those possessing the highest degree of heterozygosity. This
ic drift need not concern the investigator of cancer, however. The con-
pt of the "impurity of the gene" was given up many years ago. Muller,[50]
a recent symposium on mutations stated, "The onus of proof for the idea
a creeping variation of genes as the basis of selection would seem to lie
th those who advocate this doctrine."

Other recent concepts of genetics, such as induction and transduction,
ll probably play a very important role in cancer research for many years
come. Many significant contributions have come from such fields as
docrinology, virology, and pathology. Perhaps some day a synthesis of
e vast amount of this material will be made.

I, however, should like to reaffirm my faith in the genetic concept of the
gin of cancer originally formulated in 1926—that a change comparable to
somatic mutation was involved. This change was defined as applying to
y formed constituent of the cell, either nuclear or cytoplasmic, that could
plicate itself, affect the physiology of the cell specifically, and be trans-
tted by cell division. At that time there was no restriction to any formed
nstituent of the cell. A genic change was favored, however, and I see no
ason why the original concept should be revised.

In closing, I cite an unfamiliar quotation from the *Advancement of Learning*
Francis Bacon, foster father of the concept of the inductive method that
s meant so much to modern science: "Small and mean things conduce more
the discovery of great matters than great things to the discovery of small
atters."

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EFFECTS OF SELECTION ON CHEMICALLY INDUCED TUMORS IN MICE*

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Introduction

INDUCED TUMORS

Since the year 1775, when Percival Potts (Hueper, 1942) recognized the elationship of exposure to soot to the development of scrotal cancer appearng in chimney sweeps, clinical and industrial experiences have revealed many ther chemical agents responsible for certain types of cancer in man. Yamiiwa and Itchikawa (1918) were the first to produce malignant neoplasms xperimentally by the repeated application of coal tar to the ears of rabbits. This experiment led to many investigations of the induction of tumors in nimals (Hartwell, 1941). A very important step in an understanding of nduced tumors was reached when Murphy and Sturm (1925) showed that uccessive applications of tar to different areas of the skin in mice resulted n fewer tumors at the site of the application and, as a result, a considerable ncrease in the incidence of lung tumors. Such research emphasized the idea hat perhaps not all tumors were induced by the topical application of the arcinogen. However, following the introduction of a carcinogen into an rganism, part of it, at least, leaves the point of application and circulates hroughout the body (Vles and deCoulon, 1936). The induction of tumors t sites remote to the region of their application may be either the result of irect or indirect action of the carcinogen upon different tissues (Burrows, 932). The old concepts of local and systemic origins of tumors may thus e discussed and perhaps re-evaluated.

Many early investigators found that the application of a carcinogen to the skin of a mouse (by painting a solution of the carcinogen dissolved in benzene) led, in many cases, to skin cancer. The injection of the same carcinogen beneath the skin (in crystalline form or dissolved in a vehicle such as paraffin or sesame oil) resulted in the appearance of a fibrosarcoma. Initial credit for the discovery of the chemical nature of substances contained in coal tar and having the power to induce tumors experimentally in susceptible animals belongs to a group of English investigators, E. L. Kennaway and his colleagues (Burrows, *et al.*, 1932). About the same time, Fieser and his colleagues (Fieser, 1941) had synthesized methylcholanthrene and determined its carcinogenic properties when applied to or injected into mice. A very large number of chemical substances have since been found to have the capacity to induce tumors in certain animals.

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Due, no doubt, to the appearance of cancer in almost every mouse after the application of a carcinogen, knowledge of the molecular structure of the carcinogen has increased much faster than the genetic analysis of the nature of the responsive-to-cancer host (Kennaway and Kennaway, 1937; Fieser, 1941; Jones, 1942a, 1942b, 1942c).

Genetics of Cancer in Mice

Extensive work has been done during the last forty years on the nature of heredity in relation to the three types of tumors found in mice (spontaneous, transplanted, and induced). Like almost all quantitative characters (characters with an extended variation of phenotypes) studied thus far, the genetic nature of the initiation of tumors in mice remains an obscure question. In common with other quantitative variables, it has been shown by several investigators (but particularly by Heston, 1944) that multiple genes are involved in a susceptibility to tumors induced by chemical means. Interrelationships between the three types of tumors, however, are still not clearly understood. A wide variety of physical and chemical agents, parasites, and viruses that may somehow play a role in the transformation of a normal cell to a malignant one are known to affect an organism from its early uterine life to its death. The difficulty of controlling these agents adequately remains one stumbling block in an understanding of the genetic nature of the origin of cancer (in contrast to cancer susceptibility).

A very important outcome of early genetic work was the production of many strains of mice by hybridization, selection, and inbreeding, each strain possessing characteristics of developing tumors at certain sites in more or less constant proportions when kept under similar experimental conditions (Strong, 1942; Snell, 1941; Law, 1948). These inbred strains of mice have been valuable for cancer research (Little, 1947). In fact, the final analysis of cancer susceptibility and the origin of cancer depends upon their continued use.

Hybridization of mice of various strains, with a study of the second hybrid generation (F_2) and the backcross to the parental types has shown that the segregating genes for chemically induced cancer susceptibility are multiple (Lynch, 1927; Andervont, 1937, 1938; MacDowell *et al.*, 1941, 1945). Studies on linkage have yielded clearer results. Heston (1941, 1942a, 1942c; Heston and Deringer, 1949) has found an increase in the incidence of induced lung tumors associated with the presence of chromosomes carrying the genes for shaker, flexed tail, lethal yellow, and hairless. Other instances of linkage also have been observed (Little, 1934; Bittner, 1944; Strong, 1946). An extremely interesting field has been opened by the discovery of chemical mutagens (methylcholanthrene and the nitrogen mustards) and a high corelation between the mutagens and the carcinogens (Carr, 1948; Auerbach, 949; Demerec, 1949; Strong, 1945).

Genetic selection has been used extensively for the production of inbred trains of mice, but has not been utilized adequately for an understanding of he role of heredity in the initiation of tumors. Kreyberg (1938), however, as reported the effects of selection on the earlier and the later appearance of umors induced by tar (Saxolintjaere). Following several generations of ivergent selection, he obtained two lines that differed significantly in the ppearance of chemically induced tumors.

The purposes of the present experiment were (1) to study the effect of election on tumors induced by methylcholanthrene; (2) to produce, if possible, mice that would not develop tumors at the site of the injection of a potent carcinogen or at any other site; (3) to compare our findings with previous work on the genetic nature of cancer susceptibility and the origin of cancer; and (4) to correlate the recent findings in the field of cancer research with some fields of general biology.

Materials and Methods

ORIGIN OF pBr STRAIN

The present experiment includes observations on 2966 mice of one of the several sublines of the NHO strain, produced by a single mating of a JK♀ to a CBAN♂ in the year 1937. This subline contains the mutant genes for pink eye, brown, and nonagouti and is therefore called "pBr" (genetic con-

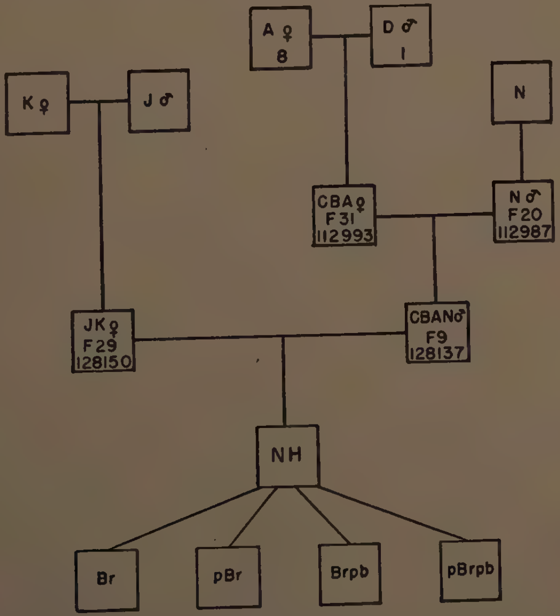

FIGURE 1. Ancestry of the pBr subline from the NHO strain.

stitution ppbbaa). The CBAN♂ used for this cross was of the ninth inbred generation from a cross of a CBA♀ to an N♂. The origins of the JK, CBA, and N strains (Strong, 1937, 1942) used for the production of the pBr and related sublines are shown in FIGURE 1.

SCOPE OF EXPERIMENT

Data are fairly complete on mice up to the twentieth generation of inbreeding following the establishment of the pBr descent. Mice of the twentieth generation born up to April 30, 1947 have all died and are taken into account in the present analysis. The experiment is being continued, and mice in the twenty-eighth generation are now under investigation.

The first 3 generations of the NHO strain consisted of 9 mice used for breeding purposes only. From the fourth generation onward, each one of 2957 mice in the pBr descent, between 60 and 70 days of age, received in its right flank a subcutaneous injection of 1 mg. of methylcholanthrene dissolved in 0.1 cc. sesame oil. The mice were allowed to breed and were permitted to live as long as they appeared to be healthy. They were sacrified when any growth appeared at the site of injection of the carcinogen or at any other location on the surface of the body. Failing a superficial tumor, the mice were sacrificed upon the appearance of emaciation, loss of weight, or any other obvious sign of serious debility. Many of the mice died without detectable tumors. They were then autopsied, and all pathological lesions were saved for sectioning and examination.

TABLE 1
NUMBER OF MICE EXCLUDED FROM ANALYSIS

Period of generations	No. of mice injected		No. of mice excluded from analysis	
	Females	Males	Females	Males
F_4–F_{12}	291	323	18	16
F_{13}–F_{16}	500	484	35	22
F_{17}–F_{20}	706	653	45	29
F_4–F_{20}	1497	1460	98	67

The data of the methylcholanthrene-injected mice are divided into three periods of successive generations, namely, F_4 to F_{12}, F_{13} to F_{16}, and F_{17} to F_{20}, for convenience of analysis. TABLE 1 shows the number of mice in each group and the mice that were excluded from a statistical calculation.

The excluded mice had either died and were eaten by their litter mates or were found in such condition that a post-mortem examination was impossible. Also a few mice had died of accidental causes or escaped early in the experiment.

The major point of selection between inclusion and exclusion for a mouse was taken to be at 60 days following the subcutaneous injection of methylholanthrene. At this time all mice were examined weekly for the presence or absence of tumors. A few mice showed induced tumors between 50 to 0 days following the injection.

LATENT PERIOD

Throughout this experiment an attempt was made to determine as accuately as possible, in days, the latent period (number of days between the njection of methylcholanthrene and the initiation of a tumor at the site of njection or elsewhere on the surface of the body) of each mouse. The time at which a suspected nodule was actually increasing in size was determined by weekly observations. Soft, flabby nodules that appeared at the site of the injection of the carcinogen were considered to be either an inflammatory reaction or due to the persistence of the sesame oil. The sizes of these early nodules were drawn on coordinate paper and a question mark was added. When the nodule became firm and increased progressively in size over a period of time the nodular mass was considered a tumor and the animal was then sacrificed. In a preliminary experiment, all methylcholanthrene-injection sites were sectioned for microscopic study to ascertain the cellular changes taking place at the time when malignant tumors were actually starting to grow. No attempt was made to study the period of survival of the mice in this series after the palpable tumors were detected. The values of latent periods are accurate for the tumors that were visible originating either at the site of the injection of the methylcholanthrene or elsewhere on the surface of the body. For mice with tumors of the internal sites, there was encountered a difficulty of getting an accurate value of latent period. Mice with internal tumors (lung, liver, stomach, or uterus) were killed when the tumors were big enough to be palpated. However, many of the internal tumors were first detected at autopsy. When a direct value of latent period was not obtainable, thirty days were subtracted from the date of killing or death in order to arrive at a rough but comparable estimate of latent periods. When a mouse developed multiple tumors, in addition to a local one, the latent period for the tumor arising at the site of the injection of methylcholanthrene or on the surface of the body was taken to be the latent period for all tumors.

SELECTION

A constant attempt was made to increase the latent periods of tumors in mice of succeeding generations by selecting young from breeders with the longest latent period. This selection experiment was part of Strong's program to obtain mice that would not give rise to malignancy in any form no matter how great the insult. In this experiment, 1 mg. of a very powerful carcinogen (methylcholanthrene), given to the mice at a very sensitive age (60 days), was considered to be sufficient. One mg. of methylcholanthrene at 1 injection is not an overwhelming dose, since a mouse receiving 2 mg. will develop a tumor somewhat earlier than a mouse receiving 1 mg. (Hollander, 1949). Up to the twelfth generation of inbreeding, only litters of those breeders that had a latent period of more than 200 days were kept. In the earlier generations of the NHO strain, which gave rise to the pBr descent without genetic selection toward resistance to chemically induced tumors, the average latent period of induced tumors was 125 days. From the thirteenth generation onward, the potentiality of each breeder was evaluated as an average of the latent periods of its female ancestry. The mean of potential latent periods of these breeders was further taken. The litters of only those breeders with a longer latent period than this mean were continued in the experiment. Once a mouse had been injected with methylcholanthrene, however, it was continued in the experiment. Only its descendants were excluded by not receiving the injection of methylcholanthrene.

The mean of the potential latent periods of breeders of the twelfth generation, which gave rise to the thirteenth and subsequent generations, was 316.6 days. The mean for the breeders of the sixteenth generation was 352.0 days. The mean for the breeders of the twentieth generation, which gave rise to the twenty-first and subsequent generations, was 410.6 days. The maximal selective latent period for any subline was 436.0 days, but these mice are beyond the scope of the present investigation.

HISTOLOGY

A total of 2544 tumors (counting all the tumors at the single site as 1) was detected at various sites in 2231 mice between the F_4 and F_{20} generations, which developed tumors after the injection of methylcholanthrene. When it was decided that a sufficient number of tumors had been examined to make possible a conclusion concerning their pathological variability and diagnosis, tumors at the site of injection with a latent period of less than 150 days were not saved. When a mouse was found dead and its tumors were not in good condition for histology, the tumors were also not saved. These were mainly lung and liver tumors, and since, in this study, all lung tumors are called adenomas and all liver tumors arising in the liver cells are called hepatomas, these tumors can be classified on gross appearance only. All other tumors were saved. The total number of tumors saved and studied for histology was 1528. Tumors were fixed in Bouin's fluid and stained with hematoxylin and eosin. Special stains were used in cases of suspected muscle tumors and in the differentiation of tumors of the forestomach.

UPKEEP OF MICE

The mice were separated at weaning age, six or less in each cage, and were given a diet of Nurishmix and lettuce, with unlimited water. They also received, weekly, a mixture of grains (wheat, oats, and sunflower seeds) and calf meal pellets. Enriched bread, soaked in fortified milk, and Squibb's cod liver oil were given to the mice twice a week. Mice used as breeders were placed in reserve following the weaning of adequate offspring to continue the next generation. All mice born in reserve were discarded at birth. Female mice in reserve were placed in other boxes containing reserve mice from time to time. When a box contained a male, however, in order to oid fighting, no other male was added. The temperature of the room in hich the mice were kept was 70° to 73° F., the humidity, 50 to 60 per cent.

Results

In order to facilitate the presentation of the results it was necessary to nalyze first the broader aspects of the experiment followed by a detailed nalysis of each suspected variable involved in the experiment.

Preliminary Analysis

Table 2 contains the data of the entire experiment included in the analysis nd their certain characteristic features. Mice are divided into three broad roups: the group "local only" includes the mice that developed tumors at he site of injection; the group "other sites" includes the mice that developed umors at other sites with or without local tumors; and the "nontumor" roup includes the mice that died and revealed no evidence of tumors at utopsy. Leukemic mice, which were few, are also included in this non-umor group. The latent period is divided into classes of 100 days each. Jnder the heading covering the latent period of the nontumor group in ABLE 2 is inserted the number of days the mice lived after the injection of he carcinogen. The shortest latent period observed for the appearance of a umor was 51 days in a male mouse. This tumor was an epidermoid car-inoma that developed at the site of the injection of methylcholanthrene. The longest latent period for any methylcholanthrene-injected mouse was 1147 days, also a male, which, at death, had 11 lung adenomas. After the njection of methylcholanthrene, a female mouse died at 947 days without evealing any tumor.

Figure 2 is a graphic representation of the data in Table 2, where the numbers of mice in each class are plotted as percentage frequencies. It shows that local tumors and tumors at other sites have a characteristic distribution of latent period. It also shows the changes in the incidence of types of tumors in the 2 sexes during the 3 successive periods of generations. The last 2 columns of Table 2 show the incidence of tumors and their percentages without taking into account the latent periods. Table 3 contains values of X^2 and the corresponding statistical significance for the 9 different combinations of data for the 2 sexes and the 3 periods of generations. The left third of this table shows the significance of the differences in the incidence of 2 sexes in the 3 periods of generations and leads to the following conclusions:

(1) Difference in the incidence of 2 broad groups of tumors and the nontumor group of the 2 sexes in the first period of F_4 to F_{12} gives a value of $X^2 = 1.2$ ($0.5 > P > 0.3$), which is not significant.

(2) Difference in the sex incidence in the second period, F_{13} to F_{16}, gives a value of $X^2 = 6.2$ ($0.05 > P > 0.02$), which is significant. This significance is due to the differences in the incidence of tumors at sites other than local and the nontumor group.

(3) The tumor incidence in the 2 sexes shows a highly significant difference in the third period, F_{17} to F_{20}. Incidence of local tumors in this period is

TABLE 2

FREQUENCY DISTRIBUTION OF MICE WITH REFERENCE TO TUMOR ONSET, LATENT PERIOD, SEX, AND GENERATION OF INBREEDING

Latent period	F_4–F_{12}								F_{13}–F_{16}								F_{17}–F_{20}							
	Females				Males				Females				Males				Females				Males			
	Local only	Other sites	Non-tumor	Total	Local only	Other sites	Non-tumor	Total	Local only	Other sites	Non-tumor	Total	Local only	Other sites	Non-tumor	Total	Local only	Other sites	Non-tumor	Total	Local only	Other sites	Non-tumor	Total
1–100	7	—	2	9	13	—	3	16	11	1	13	25	2	—	6	8	18	—	13	31	9	—	9	18
101–200	138	6	7	151	144	8	14	166	93	7	25	125	95	1	12	108	251	2	31	284	123	2	24	149
201–300	31	14	11	56	35	6	8	49	63	19	30	112	68	14	23	105	99	22	21	142	79	8	25	112
301–400	1	9	10	20	8	6	6	20	19	38	28	85	14	22	21	57	35	21	25	81	19	27	29	75
401–500	2	12	6	20	—	21	2	23	5	40	17	62	3	40	18	61	10	42	12	64	7	65	12	84
501–600	—	6	6	12	—	8	7	15	1	31	6	38	—	64	13	77	2	34	11	47	—	92	20	112
601–700	—	—	3	3	—	11	2	13	—	9	3	12	—	32	5	37	—	11	1	12	—	49	9	58
701–800	—	1	1	2	—	3	—	3	—	2	2	4	—	6	2	8	—	—	—	—	—	10	4	14
801–900	—	—	—	—	—	1	—	1	—	—	1	1	—	—	1	1	—	—	—	—	—	2	—	2
901–over	—	—	—	—	—	1	—	1	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—
Total........	179	48	46	273	200	65	42	307	192	147	126	465	182	179	101	462	415	132	114	661	237	255	132	624
Percentage...	65.5	17.7	16.8	100.0	65.2	21.1	13.7	100.0	41.3	31.6	27.1	100.0	39.5	38.7	21.8	100.0	62.7	20.0	17.3	100.0	38.0	40.8	21.2	100.0

.2 per cent in females against 38.0 per cent in males. This difference is ainly balanced by a difference in the tumor incidence at other sites.

The right-hand portion of TABLE 3 gives values of X^2 for differences in the cidence of females and males separately for the 3 possible combinations

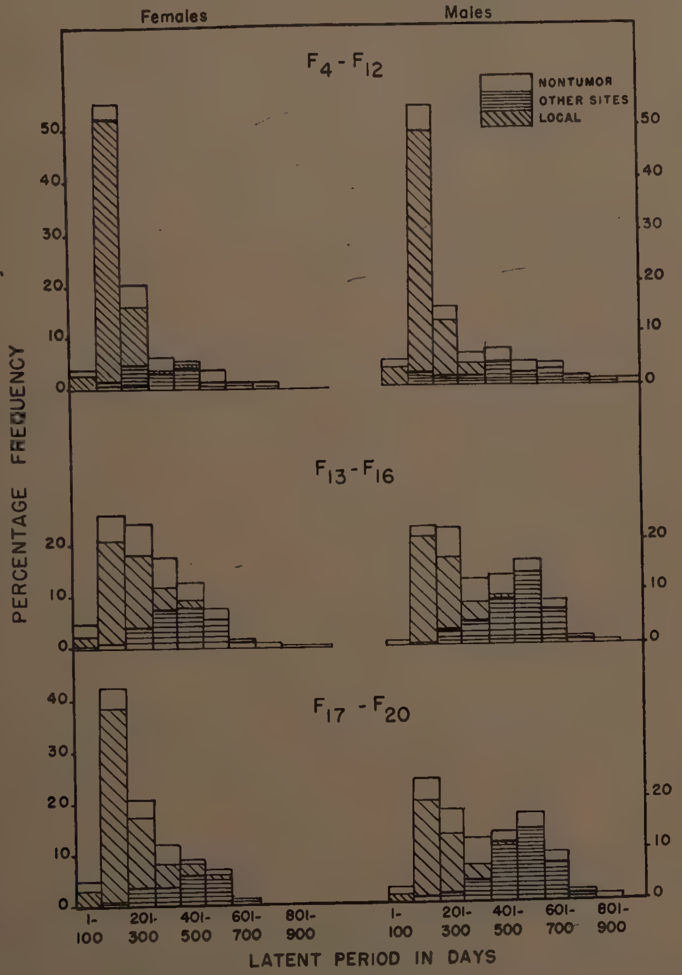

FIGURE 2. Percentage frequency of mice with their latent periods and tumor classifiation according to sites of origin.

of the 3 periods of generations, namely, I × II, II × III, and I × III. These values lead to the following conclusions:

(1) For females there is a highly significant difference in the incidence between the first and second periods and also between the second and third periods, but there is no significant difference between the first and third periods. The incidence of local tumors is 65.5 per cent in the first and 41.3 per cent in the second against 62.7 per cent in the third. This difference is

balanced by differences in the incidence of tumors at other sites and in th nontumor group.

(2) For males there is a highly significant difference between the first an second periods but, unlike the females, there is no significant differenc between the second and third. The incidence of local tumors is 65.2 pe cent in the first against 39.5 per cent in the second and 38.0 per cent in th third.

Table 3

Statistical Analysis of Tumor Onset with Reference to Sex for each Generation and the Generation for each Sex (X^2 Value and Statistical Significance)

Period	♀ × ♂		Period	Females		Males	
	x^2	P		x^2	P	x^2	P
I	1.2	.5 > P > 3	I × II	28.7	< .0001	55.7	< .0001
II	6.2	.05 > P > .02	II × III	50.8	< .0001	0.5	.8 > P > 7
III	88.0	< .0001	I × III	0.8	.7 > P > 5	61.7	< .0001

From the above observations the information about differences in the incidence of the two groups of tumors and the nontumor group could be summarized as follows. The incidence of tumors in females and males in the firs period is similar. In the second period this tumor incidence changes considerably in both the sexes, giving particularly a low value in the incidenc of local tumors. In this second period the incidence of local tumors in th two sexes is similar, but there is a significant difference in the incidence o tumors at other sites and in the nontumor group. In the third period th incidence of tumors in males remains the same as in the second period whereas the incidence of tumors in the females changes considerably onc more and is similar to the first.

Detailed Analysis

The variables examined during the course of the analysis are the gros anatomical sites at which tumors developed, their histological characteristic (wherever possible), and the latent periods measured in days. The data i the third period of F_{17} to F_{20} generations were large enough to make possibl further examination of two different families of direct lineal descent tha had had common parents in the eighth generation of inbreeding.

Site

The classification of tumor formation in Table 2 was simplified by listin in the group "local only" all mice that developed tumors only at the site o injection, by putting in the "nontumor" group all mice in which no tumo was detected at autopsy, and by putting all the rest of the mice in the grou of "other sites." By this method of classification some of the detaile ormation was lost, since the mice with local tumors that had also developed mors at other sites were grouped with the mice with "other sites." mplexity was greatly increased when "other sites" were further analyzed cording to the actual sites, because of a very large number of mice that

TABLE 4
NUMBER OF MICE WITH REFERENCE TO LOCATION OF THE TUMORS, SEX, AND GENERATIONS OF INBREEDING

Site of tumor	F_4–F_{12}		F_{13}–F_{16}		F_{17}–F_{20}	
	♀	♂	♀	♂	♀	♂
cal only	179	200	192	182	415	237
and lung	30	25	29	14	38	14
and forestomach	—	2	—	—	—	1
and liver	—	1	—	2	—	1
with lung and liver	—	1	1	1	—	3
with lung and iliac	1	—	—	—	—	—
and ovary	—	—	1	—	—	—
rface only	2	3	17	7	2	6
and lung	2	4	15	10	10	6
with lung and liver	—	—	—	—	—	2
ng only	12	23	65	124	53	155
and forestomach	1	1	1	2	10	10
and liver	—	4	—	12	2	26
and pancreas	—	—	—	—	—	1
and kidney	—	—	—	—	2	—
and heart	—	—	1	2	—	—
and ovary	—	—	—	—	3	—
and uterus	—	—	1	—	—	—
with liver and forestomach	—	—	1	—	—	3
with liver and ovary	—	—	—	—	1	—
orestomach	—	—	3	2	9	18
iver only	—	1	2	2	1	8
and ovary	—	—	1	—	—	—
and kidney	—	—	1	—	—	—
eart	—	—	—	1	—	1
vary	—	—	3	—	1	—
terus and cervix	—	—	5	—	—	—
ontumor	46	42	126	101	114	132
otal	273	307	465	462	661	624

ad multiple tumors. A mouse was classified as having multiple tumors nly when the two or more tumors were histologically different. Mice with etastatic or invasive tumors were considered to have developed only one umor but, since the mice were killed early, very few showing metastases ere encountered in the entire experiment. TABLE 4 contains an exhaustive lassification of the mice according to sites of tumors in the two sexes during he three periods of generation. This table is an extension of TABLE 2, where mice with tumors at other sites were distributed according to the sit or sites, without taking into account the latent period. A large number o mice with multiple tumors makes it difficult to comprehend the incidence o tumors at various sites. However, in order to arrive at a fairly satisfactor method of classification, the basis of analysis was changed from the numbe of mice to the number of tumors. For instance, in F_4 to F_{12}, 227 females ha an aggregate of 262 tumors and 46 died without any tumor being detected These 262 tumors are classified according to the site at which they appeared TABLE 5 contains such an analysis of the number and percentage incidenc of tumors according to the site, the sex of the mouse, and the generation during which the tumor developed.

TABLE 5
NUMBER AND FREQUENCY PERCENTAGE OF THE TUMORS IN REFERENCE TO LOCATION, SEX, AND GENERATIONS OF INBREEDING

Site of tumor	F_4–F_{12}				F_{13}–F_{16}				F_{17}–F_{20}			
	Females		Males		Females		Males		Females		Males	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Local	210	68.2	229	66.4	223	42.9	199	39.3	453	62.2	256	36.6
Surface	4	1.3	7	2.0	32	6.2	17	3.3	12	1.6	14	2.0
Forestomach	1	0.3	3	0.9	5	1.0	4	0.8	19	2.6	32	4.6
Lung	46	14.9	58	16.8	114	22.0	165	32.6	119	16.3	220	31.5
Liver			6	1.7	6	1.1	17	3.4	4	0.6	43	6.1
Misc.	1	0.3			13	2.5	3	0.6	7	1.0	2	0.3
Nontumor	46	15.0	42	12.2	126	24.3	101	20.0	114	15.7	132	18.9
Total	308	100.0	345	100.0	519	100.0	506	100.0	728	100.0	699	100.0

All mice had been injected on the right side of the body, and any tumor that developed in that area was considered a local one. A tumor arising on the left side or on any other area of the body was placed in the surface group Such tumors arose on the mouth, jaws, lips, eyelids, forehead, and regions on the front and the hind legs. Tumors were found in the forestomach; lung tumors varied in number from 1 to 20 or 25. Liver tumors (hepatomas) will be described later in the section on histology. The miscellaneous group consists of all other tumors listed on TABLE 4.

FIGURE 3 is a graphic representation of the data on percentage incidence of tumors given in TABLE 5. A consideration of the graph shows the following trends:

Local. In females the incidence of local tumors, which is 68.2 per cent in the first period, decreases to 43.0 per cent in the second and rises again to 62.2 per cent in the third. In males it decreases from 66.4 per cent in the first to 39.3 per cent in the second, and stays at 36.6 per cent in the third.

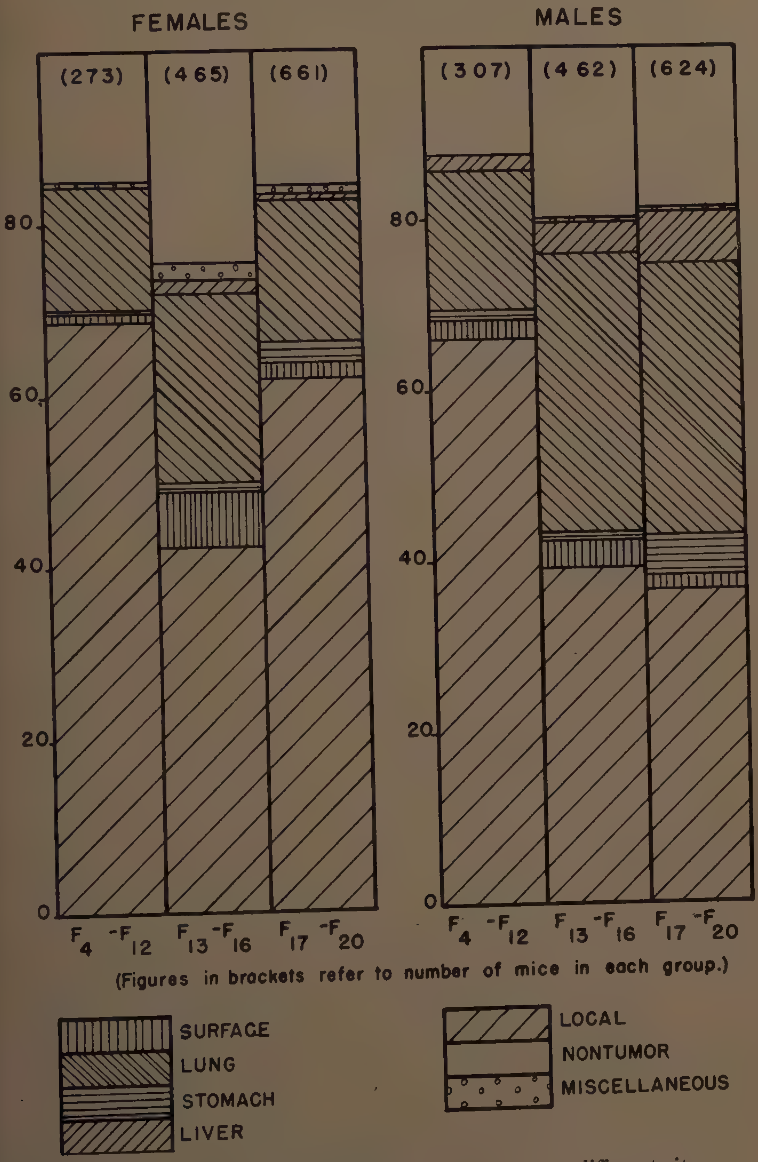

FIGURE 3. Percentage frequency of the tumors at different sites.

Surface. Both females and males show a rise in the percentage of surface tumors in the second period, the percentage in the third being similar to that in the first.

Forestomach. In both the sexes there is a rise in the incidence of forestomach tumors in the third period.

Lung. The most preponderant type in the group of tumors at sites other than the local are lung tumors. Variations in the percentage of incidenc are very large, mainly balancing the variations in the percentage of incidenc of local tumors. In females the incidence of lung tumors was 14.9 per cen in the first period, 22.0 per cent in the second, and 16.3 per cent in the third In males lung tumors developed in 16.8 per cent of the mice in the firs period, 32.6 per cent in the second, and 31.5 per cent in the third.

Liver. The occurrence of a small number of liver tumors in females wa in marked contrast to the increasing percentage of incidence in male during the three periods. In males liver tumors appeared in 1.7 per cen in the first period, 3.4 per cent in the second, and 6.2 per cent in the third accounting for 43 liver tumors of 567 tumors in males against only 4 live tumors of 614 tumors in females in the same period.

The observation based upon the preliminary analysis on slightly significan differences in the second period in the two sexes is due to the differences i the incidence of lung tumors. Highly significant differences in the incidence during the third period in the two sexes are due to differences in the incidence of local tumors, balanced by the difference in the incidence of lung and liver tumors.

Families

In F_{17} to F_{20}, of a total of 1285 mice, 403 belonged to a group of mice that were the descendants of a single mating in the fourteenth generation, and 283 mice belonged to another group that were the descendants of another

TABLE 6

NUMBERS OF MICE IN REFERENCE TO THE HISTOLOGY OF THE LOCAL TUMORS AND SEX IN THE FIRST GENERATION GROUP

Histological type					
Single type	♀	♂	Multiple type	♀	♂
Connective tissue			Fibrosarcoma and		
Fibrosarcoma	60	97	Epidermoid carcinoma	10	4
Myxosarcoma	—	2	Rhabdomyosarcoma	2	—
Undiff. sarcoma	7	9	Myxosarcoma	—	7
Pleomorphic sarcoma	—	4	Endothelioma	5	3
Muscle tissue					
Rhabdomyosarcoma	13	11	Epidermoid carcinoma and myxosarcoma	1	1
Blood vessel			Adenoma of M.G.	1	—
Angiosarcoma	1	2	Epidermoid carcinoma		
Melanosarcoma	1	1	Rhabdomyosarcoma	1	—
Epithelial tissue			Adenocarcinoma of M.G.	1	—
Epidermoid carcinoma	17	16	Carcinoma of M.G. and angioma	1	—
Anaplastic carcinoma	4	6			
Mammary gland			Total	135	163
Adenocarcinoma	6	—			
Scirrhous carcinoma	1	—			
Carcinoma	3	—			

gle mating also of the fourteenth generation. These two pairs of mice the fourteenth generation had a common parentage in the eighth genera- n. Genetic differences in the two families therefore may be the residual s after the first eight generations of inbreeding. TABLE 6 contains the nbers and percentage of incidence of tumors at different sites in these two

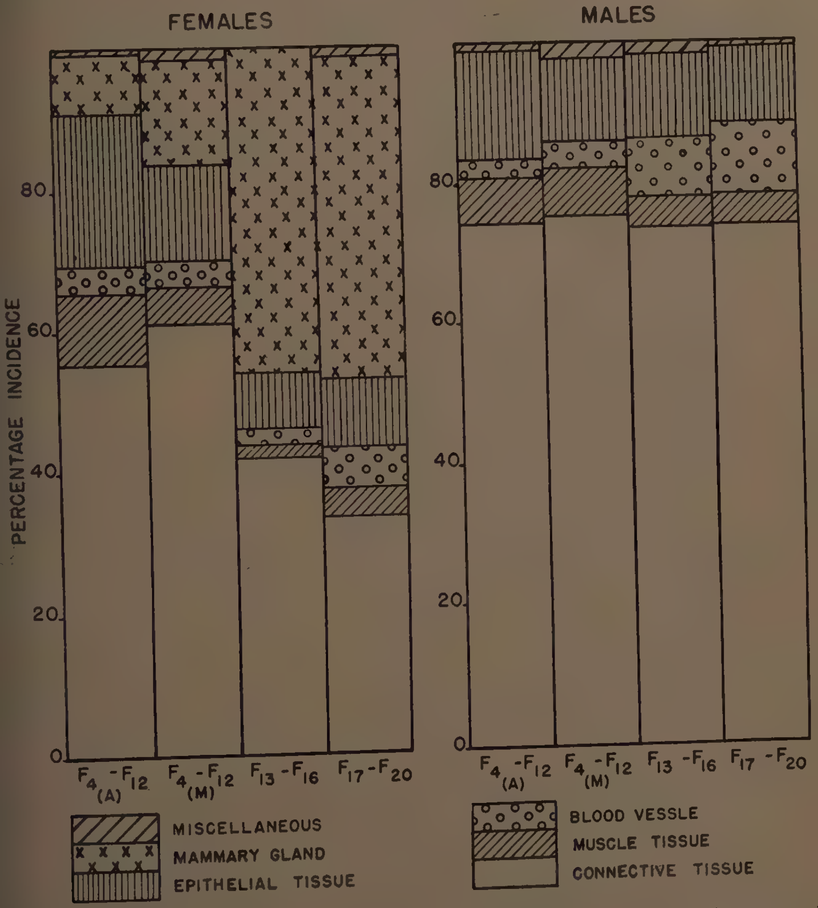

FIGURE 4. Percentage frequency of tumors at the injection site (local) in regard to istological types.

amilies. The method of analysis is based on tumors, as in TABLE 5. FIGURE is a graphic representation of these data.

Each one of the families has a characteristic incidence of tumors at dif- erent sites for each sex, whereas the incidence of the nontumor group is simi- ar for both families.

The incidence of local and surface tumors in both the sexes is higher in he first family than in the second. In females the incidence of local tumors s 59.5 per cent in family 1 against 48.8 per cent in family 2; in males the same incidence is 40.0 per cent in family 1 against 25.0 per cent in family . This difference is balanced by a higher incidence of lung and forestomac tumors in the second family. In females the incidence of lung tumors 12.1 per cent in family 1 against 26.2 per cent in family 2; in males the inc dence is 20.5 per cent in family 1 against a high value of 37.2 per cent i family 2. This difference in the specific tumor types in mice of the tw families is significant (for ♀♀ $X^2 = 13.075$, $P < 0.001$, and for ♂♂ $X^2 =$ 15.51, $P < 0.001$).

The two families were further analyzed for other variables, namely, litte seriation and latent period. The incidence of tumors at different sites i mice of the first two litters did not show a significant difference from th incidence of late litters for either sex in the two families. Difference in th means of latent period of local tumors also was not significant for eithe sex in the two families.

Histology

A large number of distinct histological types was encountered in th local tumors. Variations in histological patterns were observed at othe sites also. A brief description is given below about the histology at eac site:

Local. Of a total of 1570 tumors that developed at the site of injection 1089 were examined for histology. The first 198 local tumors were al saved, after which the local tumors with a latent period of less than 150 day were not saved. The histological classification of these 198 local tumors is given in TABLE 7. It might be mentioned that the type noted in TABLE 7 is based on the main types of cells encountered in the section. Many sections contained more than one type of cells and are included in the multiple types.

Great variability was found, not only between multiple tumors arising in a single individual, but also in the histological appearance of two sections from the same tumor. In fact, considerable variations of histology were

TABLE 7
NUMBER AND PERCENTAGE FREQUENCY OF THE TUMORS AT THE INJECTION SITE IN REFERENCE TO THE HISTOLOGY, SEX, AND GENERATION PERIODS

Histology	F_4–F_{12} (Actual)				F_4–F_{12} (Modified)				F_{13}–F_{16}				F_{17}–F_{20}			
	Females		Males		Females		Males		Females		Males		Females		Males	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Connective tissue	86	55.5	134	74.9	53	60.9	71	75.5	78	42.4	111	74.0	98	33.8	146	74.1
Muscle tissue	16	10.3	11	6.1	5	5.8	6	6.4	2	1.1	6	4.0	11	3.8	9	4.6
Blood vessel	6	3.9	5	2.8	3	3.5	4	4.3	5	2.7	13	8.7	18	6.2	19	9.6
Epithelial tissue	33	21.3	27	15.1	12	13.8	11	11.7	15	8.2	17	11.3	27	9.3	21	10.7
Mammary gland	13	8.4	—	—	13	14.9	—	—	84	45.6	—	—	133	45.9	—	—
Miscellaneous	1	0.6	2	1.1	1	1.1	2	2.1	—	—	3	2.0	3	1.0	2	1.0
Total	155	100.0	179	100.0	87	100.0	94	100.0	184	100.0	150	100.0	290	100.0	197	100.0

countered in various fields of the same tumor. It is possible to classify a mor by the prevalence of the dominant cellular arrangement. The tumor s called fibrosarcoma, however, when any fibroblastic arrangement of the lls could be indicated.

Surface. Of 86 tumors that developed on the surface of the body at sites her than local, 60 were examined for histology. Their types were similar those obtained in the local tumors.

Forestomach. Of 64 tumors of the forestomach, 52 were examined for stology. They showed two main types: epidermoid carcinoma and spindle ll neoplasm. A third type was a mixed type of these two. The incidence epidermoid carcinomas was 11 in females and 16 in males, the incidence spindle cell neoplasm was 8 in females, 9 in males, and the incidence of the ixed type was 3 in females and 5 in males.

Lung. Of 722 lung tumors 277 were examined for histology. No attempt as made to classify the histological variations in these tumors.

Liver. Of 76 liver tumors 40 were examined for histology; 33 were hepa- mas, 5 histiocytomas, and 3 reticulocell sarcomas.

Miscellaneous. This group contains the tumors of the uterus, ovary, idney, and heart. Of 3 tumors of the uterus examined, 2 were hemangiomas nd 1 was leiomyosarcoma. All 3 ovarian tumors were granulosa cell tumors, nd the 2 kidney tumors were hypernephromas. Two heart tumors examined ere secondary to mesothelioma, arising from either the surface of the lung r from the pleura.

No data of the various histological types were sufficient for further sta- istical analysis except the local tumors, and here the picture of the incidence f histological types was complicated by the fact that the tumors with a atent period of less than 150 days were not saved during the later part of the xperiment. In view of the variations in the incidence of local tumors in the periods of generations, it was important to see whether variations were also aking place in the incidence of different histological types in the 2 sexes during he 3 periods. An attempt was made (TABLE 8) to compare relative varia- ions in the histological types of local tumors. The difficulty of grouping the multiple histological types was solved partly by effecting a classification on he basis of histological types instead of tumors. Some new types appeared n the second and third periods, and all were conveniently classified in the ive broad types shown in the table. The data shown in the column of F_4 to F_{12} (A = actual) are the exhaustive data shown in TABLE 7. For a rough comparison with the other 2 periods, these data are modified by not taking nto account the local tumors with a latent period of less than 150 days. FIGURE 5 is a graphic representation of these data and suggests the following conclusions:

(1) A comparison of F_4 to F_{12} (A) in the two sexes shows that the percent- age of incidence of histological types differs in the two sexes. The incidence of connective tissue tumors is 55.5 per cent in females and 74.9 per cent in males.

(2) In females the incidence of mammary gland tumors increases from 14.9 per cent in the first period to 45.6 per cent in the second and stays as

TABLE 8
FREQUENCY DISTRIBUTION AND MEANS OF THE LATENT PERIODS OF THE TUMORS AT VARIOUS LOCATIONS
(SEXES AND GENERATION GROUPS COMBINED)

Site of tumor	Latent period																		Total	Mean
	1 50	51 100	101 150	151 200	201 250	251 300	301 350	351 400	401 450	451 500	501 550	551 600	601 650	651 700	701 750	751 800	801 850	851 +		
Local only	—	40	246	316	199	110	59	28	15	10	3	—	—	—	—	—	—	—	1026	201.2 ± 2.5
Surface	—	—	—	2	3	3	8	11	19	10	10	11	4	2	1	—	—	—	84	447.0 ± 12.6
Forestomach	—	—	—	—	—	2	2	8	13	10	7	9	4	2	—	—	—	—	57	477.7 ± 12.7
Lung only	—	1	2	1	11	7	16	28	58	65	81	68	53	19	10	10	1	1	432	510.1 ± 5.8
Liver	—	—	—	—	—	—	2	3	2	4	10	14	11	8	3	1	—	—	58	569.9 ± 13.2
Nontumor	16	30	50	63	65	53	62	57	41	26	36	27	13	10	5	4	2	1	561	319.0 ± 7.2

9 per cent in the third. The incidence of connective tissue tumors drops
m 60.9 per cent in the first to 42.4 per cent in the second and 33.8 per cent
the third. Mammary gland tumors were entirely restricted to the
ales.

3) In males percentage incidence of histological types remains more or
s the same in the three periods of generations.

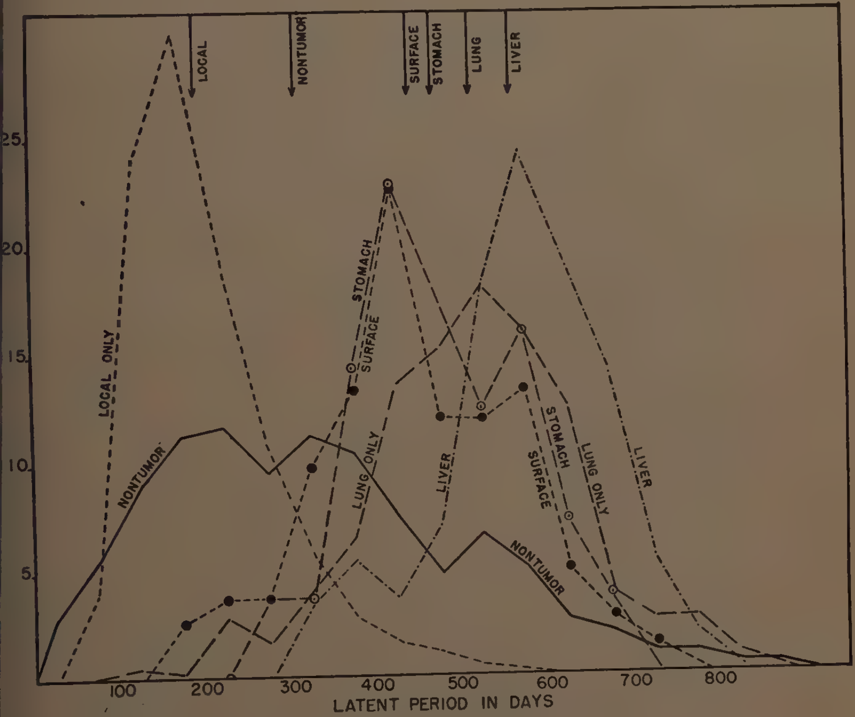

FIGURE 5. Percentage frequency of the latent periods for tumors at various sites.

FIGURE 3 shows that changes in the percentage incidence of local tumors
1 the three periods of generations are not related to the changes in the per-
entage incidence of their histological types.

Latent Period

As mentioned previously, an attempt was made to determine as accurately
s possible the latent period of tumors at different sites. It was possible
o get an accurate value of the latent period for local and surface tumors.
n other instances, when a direct value was not available, 30 days were sub-
racted from the date the animal was killed or found dead. TABLE 9 con-
ains frequency distributions of latent periods for local tumors in classes of
0 days at 5 different sites and the nontumor group. Data for the 2 sexes
nd the 3 periods of generations are combined together. Local and lung

TABLE 9

FREQUENCY DISTRIBUTION AND THE MEAN LATENT PERIOD FOR TUMORS AT THE INJECTION SITE IN REGARD TO SEX AND GENERATION GROUPS

Period of generations	Sex	Latent period 51 75	76 100	101 125	126 150	151 175	176 200	201 225	226 250	251 275	276 300	301 325	326 350	351 375	376 400	401 425	426 450	451 475	476 500	551 +	Total	Mean
F_4–F_{12}	♀	1	6	38	45	31	24	13	11	3	4	—	—	—	1	1	—	—	1	—	179	163.1 ± 4.3
	♂	3	10	42	48	34	20	16	10	7	2	4	1	3	—	—	—	—	—	—	200	162.6 ± 4.3
F_{13}–F_{16}	♀	1	10	14	18	35	26	24	18	8	13	9	6	2	2	2	2	1	—	1	192	207.2 ± 5.8
	♂	—	2	12	21	34	28	16	23	12	17	8	4	—	2	—	—	3	—	—	182	209.3 ± 5.2
F_{17}–F_{20}	♀	5	13	47	83	79	42	38	23	23	15	12	10	7	6	3	1	2	4	2	415	193.5 ± 4.3
	♂	1	8	19	32	39	33	30	27	13	9	4	6	2	7	3	4	—	—	—	237	203.9 ± 4.8

ups contain the mice with local and lung tumors only. Surface, fore-mach, and liver groups contain mice with those respective sites, together h mice having lung tumors. All other mice with multiple tumors are

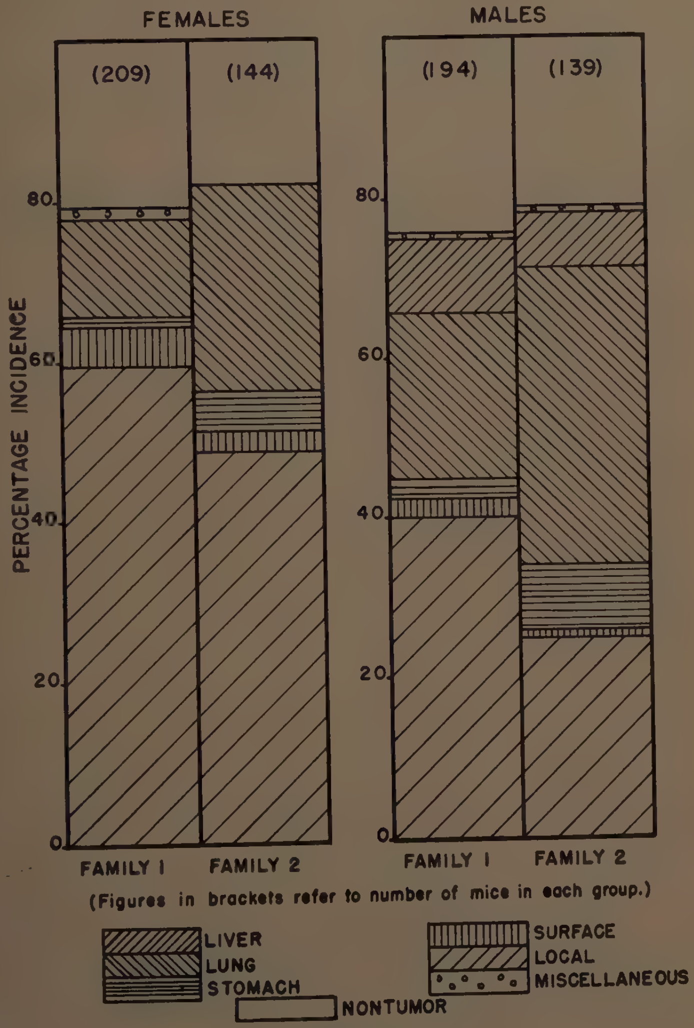

FIGURE 6. Percentage frequency of tumors at various sites for both families (numbers parentheses are the numbers of mice in each group).

xcluded from these distributions (FIGURE 6). In order to avoid complexity, ontinuous curves are drawn instead of histograms.

The last column of TABLE 9 gives mean values with their standard error of atent period of each one of these sites. It might be noted that each site as its characteristic value different from the rest. The nontumor group as an average survival time of 319.0 days after the injection.

TABLE 10

FREQUENCY DISTRIBUTION AND THE MEAN LATENT PERIOD FOR TUMORS AT THE INJECTION SITE IN REGARD TO SEX AND GENERATION GROUPS

Sex	Period of generations	Latent period																	Total	Mean
		51 100	101 150	151 200	201 250	251 300	301 350	351 400	401 450	451 500	501 550	551 600	601 650	651 700	701 750	751 800	801 850	851 +		
Females	F_4–F_{12}	—	—	—	—	1	2	1	2	2	1	2	—	—	1	—	—	—	12	458.3 ± 41.0
	F_{13}–F_{16}	1	1	—	3	2	8	5	9	11	12	6	3	2	1	1	—	—	65	452.7 ± 16.7
	F_{17}–F_{20}	—	1	—	4	—	2	4	8	9	13	6	5	1	—	—	—	—	53	469.3 ± 16.8
	F_4–F_{20}	1	2	—	7	3	12	10	19	22	26	14	8	3	2	1	—	—	130	460.4 ± 11.1
Males	F_4–F_{12}	—	—	1	—	—	—	—	4	5	1	3	5	1	1	1	1	—	23	548.9 ± 29.1
	F_{13}–F_{16}	—	—	—	2	2	2	11	18	14	20	25	18	6	3	3	—	—	124	522.2 ± 9.3
	F_{17}–F_{20}	—	—	—	2	2	2	7	17	24	34	26	22	9	4	5	—	1	155	536.3 ± 8.6
	F_4–F_{20}	—	—	1	4	4	4	18	39	43	55	54	45	16	8	9	1	1	302	531.5 + 6.4

The numbers of mice in the surface, forestomach, and liver groups are too all for further detailed analysis. The numbers of mice in the local, lung, d nontumor groups are sufficiently large and are analyzed further. Mean ues of the nontumor group in the two sexes during the three periods of nerations do not show significant differences. Frequency distribution and an values of local and lung tumors in the two sexes in the three periods e given in TABLES 10 and 11.

TABLE 11
NUMBER OF MICE AND PERCENTAGE FREQUENCY OF THE TUMORS IN REGARD TO SITE AND SEX FOR THE TWO DIFFERENT FAMILIES, IN THE THIRD GENERATION PERIOD ONLY

Site of tumor	Family 1				Family 2			
	Females		Males		Females		Males	
	No.	%	No.	%	No.	%	No.	%
ocal	138	59.5	84	40.0	80	48.8	41	25.0
rface	12	5.2	5	2.4	4	2.4	1	0.6
orestomach	2	0.8	6	2.8	8	4.9	14	8.5
ung	28	12.1	43	20.5	43	26.2	61	37.2
ver	—	—	19	9.0	—	—	11	6.7
isc	3	1.3	1	0.5	—	—	1	0.6
ontumor	49	21.1	52	24.8	29	17.7	35	21.4
otal	232	100.0	210	100.0	164	100.0	164	100.0

Frequency distribution of local tumors is given in classes of 25 days. Differences in the means of latent periods between the 2 sexes are not significant in any 1 of the 3 periods. Means of the 2 sexes, however, are significantly lower in the first period than in the second and third periods. This finding has a particular significance in view of the changes in the total ncidence of local tumors with uncorrelated changes in the percentage incilence of their histological types. A pertinent point was to find whether this ncrease was a lengthening of the latent period of some of the histological types or whether it was due to a change in the percentage incidence of histological ypes. From an analysis of the first 198 local tumors that were all saved, t was found that the difference in the mean latent period of epidermoid arcinoma (150.8 ± 7.0 days) and of fibrosarcoma (167.4 ± 3.5 days) is significant ($t = 2.11$, $0.05 > P > 0.02$). In view of large changes in the percentage incidence of histological types of local tumors in females and practically no change in males, it is not possible to decide this point.

TABLE 11 shows the frequency distribution of lung tumors in classes of 50 days. Means of the latent period for males in each of the 3 periods are higher than the means for females, and this difference between the 2 sex is significant. Differences between the periods of generations for each s are not significant.

General Discussion

METHOD OF ANALYSIS

Investigations concerning the genetic analysis of induced tumors in mi have usually been hindered by the difficulty of handling the data adequatel The usual method has been to record the percentage incidence of tumors at certain time after the injection of a carcinogen. Heston (1944) has trie to use the number of nodules in the lungs of mice as a measure of variabilit Curtis *et al.* (1934) have used the same technique of counting nodules in th analysis of tumors in the liver of rats bearing cysticerci of the cat tapewor No doubt these methods have given a comparative idea of the potency carcinogens or a comparative idea of the susceptibility of mice and rats different strains. These investigators fail, however, to give a precise idea the effect of carcinogen in terms of latent period. They also fail to take int account a large amount of information as to other effects in mice that did n develop tumors up to a certain point of time.

The method of exclusion of animals that did not develop a tumor is als probably not wise, since it distorts the relative importance of carcinogen an host in the development of a specific type of induced tumor.

Kreyberg (1938) has adopted a slightly different method by giving, fo each month, the proportion of animals with tumors expressed as a percentag of the number of animals living at the moment, plus the animals that had die of a similar tumor (cumulative data). This method partially meets th second objection but, like the other methods, it overlooks the first.

A large number of variables is now known to influence the onset of induce tumors in mice and must be taken into consideration in the genetic analysi of such tumors.

The method of analysis adopted for the present data was devised to mee the objections and, at the same time, to take into consideration these othe variables. It might be noted that the experiment was not planned to stud the probable effect of segregating genes in the F_1 and F_2 or the backcros generations upon the appearance of the induced tumor. On the contrary the first three generations of the experiment were utilized only for furthe breeding without the injection of a carcinogen. The method of analysis i therefore of an entirely statistical nature and originally was adopted for th purpose of studying the effect of selection toward resistance for induce tumors in mice of the F_4 to F_{20} generations. The percentage of homozygosity theoretically increases from 75 per cent in the fourth generation t about 99 per cent in the twentieth generation, and is more than 95 per cen from the twelfth generation onward (East and Jones, 1919).

The generations of experimental animals are classified into the followin three periods: (1) F_4 to F_{12}, (2) F_{13} to F_{16}, and (3) F_{17} to F_{20}. This procedur leaves adequate numbers of mice in each period and, at the same time, per the study of the possible trend of genetic selection. It is known that atent period and the survival time are two different variables; it was ssary to decide what was to be measured under the limitations imposed he experimental difficulties. Differences have recently been observed e behavior of these two variables in relation to characteristics of malig- y. Experiments are in progress at this laboratory to determine whether e is any correlation between the two.

rom the point of view of the effect of carcinogen upon the origin of tumors, as thought, however, that the important genetic variable is the latent od that measures, in terms of days, the time required for the carcinogen urn a normal cell into malignancy. The factors that would determine the th of a tumor and the survival of the animal after the initiation of the or are probably of a different nature than those that would determine initiation of the tumor. As mentioned previously, it is possible to get an rate value of latent period of a tumor that is on the surface of the body. tumors of the internal sites, however, it is not possible to determine the t time at which the tumor originated. In FIGURE 6 it seems clear that ranges of variation of latent periods for forestomach and liver tumors fairly small, and an estimate based on subtraction of thirty days from the e of killing or death of the animal might be close to the correct value. he case of lung tumors, with slower growth potentialities, there is a con- rably larger range of latent periods; here it seems that the survival time ht be considerably longer than thirty days. In the absence of a reliable e of survival time of lung tumors, thirty days also were subtracted in case of lung tumors in order to arrive at a comparative value of latent iod. When comparing the absolute values of latent periods of different s, this point regarding the lung tumors should be kept in mind.

Other variables that were necessarily taken into account in the present lysis are the sexes, the anatomical sites, the histological types, litter iations, and different families in the strain. There were difficulties in ling with the anatomical sites and the histological types. A single injec- n of carcinogen sometimes produces a series of multiple tumors at different es; sometimes multiple histological types appear at the site of injection d other sites. Theoretically, for an adequate genetic analysis, it would be cessary to consider a tumor instead of a mouse as a unit of classification en considering the effect of a carcinogen on the sites, and to consider a tological type as a unit of classification when considering the effect on the tological types (a unit character?). This procedure is impossible here. us, the following three different levels of classification are possible: (1) e mouse, (2) the anatomical site, and (3) the histological type. This is cessary in order to consider the effect of a carcinogen upon selection at ferent levels of continued inbreeding.

The method adopted for the present analysis makes possible the visualiza- n of the effect of the carcinogen on either the mouse, the anatomical sites, the histological types of tumors in terms of probability. This procedure kes into account, singly or jointly, the percentage incidence of tumors or e latent periods for a specific type of tumor. The effectiveness of this

method is made clear by a concrete example. The first column of TABL
would mean that, for a female mouse born in the F_4 to F_{12} generations, t
probability that it will develop a local tumor at the site of the injection of t
carcinogen is 0.682; a lung tumor, 0.149, and so on; and that the probabil
of its dying without having any tumor is 0.150. A similar interpretati
can be given to other columns of the table. The values of TABLE 7 give t
probability of different histological types when a local tumor also has dev
oped. For instance, the first column would mean that, for a female mou
born in the F_4 to F_{12} generations and developing a local tumor, the probabili
that the tumor is arising from connective tissue is 0.555; from muscle tissu
0.103; and so on. This method thus offers the advantage of utilizing
the information in a precise way.

DISCUSSION OF THE RESULTS

Variations in the incidence of tumors at various anatomical sites a
histological types are given in full detail in the preceding analysis. A
attempt is made below to coordinate the results of latent periods and s
differences. Variations in the incidence, latent period, and sex differen
are the simultaneous results of the combined effects of carcinogen and sele
tion and should therefore be visualized as such.

Latent Period

This preliminary analysis (TABLE 2 and FIGURE 2) shows that the effect
selection toward resistance to induced tumor is to increase the average late
period jointly for *all types* of tumors. Further analysis, however, revea
certain additional interesting points.

The result of selection toward resistance on local tumors (TABLE 10) wa
to increase the latent period of all tumors for mice of the second and th
third periods of generations in both the sexes. The only estimates availab
for the latent period of histological types of tumors are for epidermoid ca
cinoma and fibrosarcoma, and there is a significant difference in the late
period of the two. In view of the fact that the tumors below a latent peric
of 150 days were not saved during the later part of the experiment, it was n
possible to decide whether the increase of average latent period is due
variations in the incidence of specific types of tumors or to the lengthening
the latent period for all types.

TABLE 11 gives the latent periods of lung tumors in the two sexes durin
the three successive periods of generations. This series includes the late
periods of mice that had lung tumors only. In almost all these cases mi
had died and the tumors were detected at autopsy. The values of late
periods are therefore comparable to one another, although they are n
absolute. The mean values of the latent period show that there are no si
nificant changes in the latent period during the three periods of generatio
in the same sex; this is true for both the sexes. Thus, the selection
descendants of breeders with longest latent periods did *not* result in lengthe

he latent period of lung tumors. There is, however, a significant differ- in the latent period for lung tumors in mice of the two sexes, and this nsistent in all the three periods of generations.

ne values of latent periods in TABLE 9 (FIGURE 6) show that there are iderable variations in the latent periods of different sites under the con- ns of the present experiment.

ne findings regarding the latent period could be summarized as follows. e are considerable differences in the mean values of latent periods for ors at different sites. The mean value is 201 days for tumors at the site jection, 447 days for surface tumors, 478 days for tumors of the fore- ach, 510 days for lung tumors, and 570 days for liver tumors. There minor variations in the mean values of latent periods of different his- gical types at various sites. For local tumors the mean value of the nt period for epidermoid carcinoma is 151 days and for fibrosarcoma is days. There are also small variations in the 2 sexes at different sites. lung tumors the mean value for females is 460 days; for males it is 532 s. Probably there is *no* effect of selection toward greater resistance to ors in lengthening the latent period of any specific type of tumor.

Sex Difference

reliminary analysis (TABLE 3) shows that there is no significant difference he incidence of tumors in the two sexes in the first period of generations. ere is a slight sex difference in the second and a highly significant sex differ- e in the third. Although there is no significant difference in the incidence ocal tumors in the first and second periods, the percentage incidence of tological types of local tumors differs in the two sexes. The incidence of nective tissue tumors is 55.5 per cent in females and 74.9 per cent in les in the first period. The incidence for local tumors is 42.4 per cent in nales and 74.0 per cent in males in the second period. A small difference the second period in the two sexes is due to the difference in the incidence lung tumors and the nontumor group. In the third period the incidence local tumors in females is 62.2 per cent and in males 36.6 per cent. The idence of connective tissue tumors in this period is 33.8 per cent of the al tumors in females and 74.1 per cent in males. The percentage incidence histological types of local tumors does not show significant variations in three periods in males, but shows considerable variations in females. e incidence of mammary gland tumors in females increases from 14.9 per nt in the first to 45.6 per cent in the second and to 45.9 per cent in the third riod.

The incidence of liver tumors shows differences in the two sexes. In nales it is very low in all of the three periods of generations; in males it creases from 1.7 per cent in the first period to 3.4 per cent in the second d to 6.1 per cent in the third. The differences in the incidence of tumors different sites in the two sexes are confirmed by the analysis of the families.

The findings regarding the sex difference could be summarized as follows. here are considerable differences in the incidence of tumors at different anatomical sites, and also in the histological types of tumors in the two se The only major change in tumor incidence in the males during the experim is between the first period and the second, reflected in a large decrease in incidence of local tumors. The incidence of histological types of local tum in males does not show much variation. Females show considerable chan both in tumors in different anatomical sites and in histological types tumors during all three periods. There are small differences in the m value of the latent period between the two sexes. The incidence of li tumors in the two sexes also shows striking differences. Mammary gl tumors are restricted to the females.

Before giving final conclusions based on these results, it is necessar consider certain information regarding the genetic interrelationship betw the spontaneous, the transplanted and the induced tumors, and the mec nism of action of the carcinogen.

GENETICS OF CANCER IN MICE

As mentioned in the introduction, there are three different levels at wh genetic studies of cancer in mice are being carried out: (1) the spontane tumor, (2) the transplanted tumor, and (3) the induced tumor. Little (194 has published a comprehensive bibliography of the subject under these th headings. An attempt is made below to take into account the most pertine facts about each level and to consider the probable genetic interrelationsh between them.

Spontaneous Tumors

A general picture of the incidence of various types of spontaneous tum in a large population of mice is given in a series of papers by Sly, Holm and Wells (see Little, 1947). However, the most important source of inform tion on the spontaneous tumors in mice has been obtained by the use of t inbred strains of mice now available. There are two main features reveal by the study of the incidence of spontaneous tumors in mice:

(1) It has been possible to establish a high percentage incidence of tum at certain sites in mice of some strains by hybridization, selection, a inbreeding. For instance, the mice of strain C3H have an incidence of ab 90 per cent of mammary gland tumors in breeding females; the mice of t A strain have an incidence of 80 to 90 per cent of lung tumors; and the mi of strain C58 have an incidence of 90 per cent of leukemia.

(2) Strains are available in which there is a gradation of the percenta incidence of tumors at any one of these sites from a high to an extremely l value. For instance, the incidence of mammary gland tumors is about per cent in C3H, about 60 per cent in dba, only about 9 per cent in N, a less than 1 per cent in JK and F strain mice.

The clearest evidence of the genetic nature of susceptibility to spontaneo lung tumors has been supplied by Lynch (1941) and by Heston (1942 Here it appears that multiple genes are involved. It has not been demo strated, however, whether a complement of genes is responsible for the co

transmission of lung tumor susceptibility or whether some other influ-
changes this intrinsic susceptibility.
ncerning the problem of the adenocarcinoma of the mammary gland,
ıer (1940) clearly states that three influences are known to affect the
ence of breast tumors in mice: (1) an inherited predisposition, (2) a
ıonal influence, and (3) the milk agent. In addition to these three
cies, however, a somatic mutation also may be involved in the origin of
st tumors in mice. This concept has been maintained particularly by
ng (1926a, 1926b) and by Bauer (1928).
ıe researches on leukemia by MacDowell *et al.* (1945), by Cole and Furth
1), and by Kirschbaum and Strong (1939, 1942) indicate that multiple
s are involved in tumor susceptibility. MacDowell *et al.* (1947), how-
, reported "blending" inheritance for leukemia. It is difficult, therefore,
not impossible to explain all the facts of susceptibility to leukemia in mice
rms of gene action.

Transplanted Tumors

ommon features of the transplantation of tumor experiments could be
marized thus: spontaneous tumors derived from mice of an inbred strain
be transplanted to all members of such a strain, but not to individuals
n unrelated strain. A few exceptions have been observed. In the course
erial transplantation, changes occur in the transplantability of a tumor
, henceforth, it could be transplanted into mice of more strains and, at
es, could be transplanted into mice of almost any strain (Strong, 1926a).
ther, a tumor derived from a mouse of any inbred strain also could be
ısplanted to all the F_1 hybrids produced by a cross of mice of the parent
in with mice of any other inbred strain (dominant inheritance). In the
and the backcross to the parental types the percentage of successful trans-
nts might vary between 75 and 5 per cent. However, the percentage of
ceptible mice of the F_2 generation is not always mathematically consistent
h the ratio obtained in the backcross to the recessive parent.
udden changes in the transplantability of tumors were first reported in
6 by Strong and were used by him to support the concept that mutations
y be taking place in the tumor cell.
Tumors induced by chemical means show, on the average, greater capacities
grow, not only in other mice of the same strain from which they originated,
t also in mice of totally unrelated strains. The mechanism of transmission
susceptibility to the induced tumor probably does not differ fundamentally
m the mechanism involved in the transplantation of the spontaneous
nor, although the problem has not been adequately investigated.
Susceptibility to tumors is a term that has caused some confusion in con-
eration of the probable role of heredity in tumor development. It has
en used in consideration of the incidence of spontaneous tumors as well as
connection with the percentage of successful transplants. It should be
arly understood, however, that they may be entirely unrelated phenomena.
e mechanism involved in the origin of a malignant tumor from tissue may
quite different from that involved in the growth of a tumor and the sub-

sequent fate of the host as a result of the growth of the malignancy. .
spontaneous tumor is a result of a normal cell (the argument is not affec
if there is more than one cell) of an organism turning into malignancy, :
its subsequent growth and reproduction by cell divison. A transplan
tumor is the result of a malignant cell transferred to another organism.
host offers only an adequate environment for its growth and reproducti
A tumor is, therefore, the result of at least the following two process
(1) the initiation of a tumor, that is, transformation of a normal cell int
malignant one, and (2) growth and reproduction of this malignant c
Spontaneous tumors as found in mice of inbred strains are the combi
results of these two (probably) independent processes, whereas transplan
tumors, whether they are grown in a subcutaneous tissue, anterior chambe
the eye, or anywhere else, are concerned entirely with the second process
growth and reproduction.

Induced Tumors

Direct genetic experiments on the relationships between spontaneous a
induced tumors are complicated by several factors. Lynch (1936) show
that the susceptibility to induced sarcomas is not correlated with spontane
mammary gland tumors. Andervont (1939) showed that, in C3H and
strain mice, susceptibility to induced liver growths is not correlated w
susceptibility to spontaneous hepatomas. Heston (1942b; Heston a
Deringer, 1947), however, presented evidence to suggest that the effect o
carcinogen is to enhance the already existing intrinsic susceptibility
spontaneous tumors at least in the case of lung tumors. The most dire
role of heredity in induced tumors was demonstrated by Heston (1941, 194
1942c; Heston and Deringer, 1949) by showing an increased incidence
lung tumors associated with the presence of chromosomes carrying genes f
shaker, flexed tail, lethal yellow, and hairless. He has shown that the la
two genes also are associated with increased body weight; this observation
in line with the suggestion that the effect of certain genes on susceptibili
to lung tumors may be related to a more general effect of the genes on grow
or on fat metabolism.

Interrelationship

For an understanding of the interrelationship of the three levels of geneti
of cancer in mice, it would be helpful to view the problem from several angl
As mentioned before, a clear distinction should be made between the proce
of transformation of a normal cell into a malignant one and the subseque
growth and reproduction of such a cell. There should also be a clear distin
tion between the three ways in which heredity can possibly play a ro
(1) the presence of genes in the cell which, by their action through metabo
products or otherwise, would transform it directly into malignancy fro
within the cell; (2) the role of heredity of a secondary nature in determini
the degree of susceptibility of the cell to its transformation into malignan
by the action of external agents, including the products (for example, ho
mones) of the same organism to which the cell belongs (such products mig

hately be under gene control); and (3) an intermediate condition between
irst two, that is, the possible presence of genes within the cell that would
g about the transformation of the cell to malignancy if these genes were
ed by external agents to reach what is described as a certain threshold.
ransplantation is concerned primarily with the growth and reproduction
malignant cell and not with the transformation of a normal cell into
gnancy. Spontaneous and induced tumors concern primarily the trans-
hation of a normal cell into a malignant one; it would be helpful to con-
r the role of heredity from the approach suggested above to understand
probable interrelationship between them.

he concept of threshold is extensively used in several branches of bio-
cal research, particularly in the genetic analysis of quantitative char-
rs and in the production of X-ray and ultraviolet-light mutations. Its
ning and application as far as malignancy is concerned are mentioned
viously. There are inherent difficulties in demonstrating the existence
uch genes, as their effects are dependent upon external agencies. The
e concept might be looked upon from the second point of view by a slight
dification, namely, that malignant transformation is brought about by
ain external agents acting on a cell whose susceptibility to such a trans-
mation is controlled by certain genes.

tudy of the spontaneous tumor would probably offer the best material for
ertaining whether there are any genes in the cell that bring about its
lignant transformation. Extensive work on spontaneous mammary
nd tumors (for review see National Cancer Institute Staff, 1945) has
wn several interesting features. The C3H strain has an incidence of
6 per cent in breeding females at an average age of 8.1 months (Andervont,
9). Lathrop and Loeb (1916) showed that ovariectomy at an early age
her completely inhibited or greatly delayed the appearance of mammary
nors. Loeb (1919, 1924) showed further that the influence of ovarian
rmone was a quantitatively graded one. Lathrop and Loeb (1918) had
lized from the study of reciprocal hybrids that the mother exerted a large
luence on the incidence of mammary gland tumors. Bittner (1936)
owed that this maternal influence was due to an agent present in the
other's milk. When female mice of a strain with a high tumor incidence
re nursed by foster mothers belonging to a strain with a low tumor inci-
nce, the foster-nursed animals showed a low tumor rate (Heston, 1948).
contrast, mice of the C strain (the Bagg albino) that had a low incidence
spontaneous mammary gland tumors (1.1 per cent at 24 to 29 months—
imkin and Andervont, 1942) turned into a strain with a high incidence of
ammary gland tumors after the mice of one generation were suckled by
H mothers (Andervont, 1949). This suggests that a constant proportion
the mammary gland tumors in C3H breeders is a result of two different
muli, one an overproduction of an ovarian hormone and the other a milk
ent that has probably been picked up by mice of the C3H strain at some
age. This milk influence has a capacity to reproduce and metabolize in
ice of certain strains. Overproduction of the hormone and capacity to
produce and metabolize the agent might ultimately be under gene control

in C3H mice, but the direct effect of heredity is probably on the susceptibi of the mammary gland tissue.

Recent work has disclosed the fact that the milk influence in a nucleop tein. Basing his opinion on the observation of Samuels *et al.* (1947) t mice having the milk influence show a disturbed 17-ketosteroid output in urine, Bittner (1948, 1949) has expressed the opinion that the milk influe may be of the nature of an enzyme. However, Strong has expressed opinion that the milk influence, being of a nucleoprotein nature, may capable of combining with the nucleoprotein units (the genes) within cell and thus may lead to a disturbed cellular physiology by an altered act and reaction system of spatially related nucleoproteins. This disturbed lular behavior may lead to uncontrolled growth and possibly to cancer.

Spontaneous leukemia studies (Murphy, 1944; MacDowell *et al.*, 1947) a suggest influences by external agents. No such external agents have, ho ever, been detected as affecting the origin of spontaneous lung tumors.

Studies on linkage should be considered here to determine whether th give any evidence of direct action of genes. As mentioned before, there positive evidence that the two genes, lethal yellow and hairless, which associated with an increased incidence of lung tumors, are also associat with increased body weight and therefore this would suggest a possibility action through some physiological avenue. However, it is still not cle whether these two genes, lethal yellow and hairless, are the genes that inf ence lung tumor susceptibility or only show linkage relationship to two oth genes that do. This point could be determined only by the building up isogenic strains in which lethal yellow or hairless alone are segregatin This has not been done.

The possible relationship between the spontaneous and the induced tum would thus depend upon our knowledge of whether the genes play a dire role on the origin of cancer or only respond to the external agents involved malignant transformations. If the external agents play a direct role, spontaneous tumors also would be induced. If, however, genes play a dire role, a concept of the threshold effect would give an explanation for the orig of the induced tumor.

The problem of cellular differentiation might be considered. The persi ence of physiological differentiation in morphogenesis suggests involveme of cellular heredity. Even malignant cells preserve their tissue specifici after a long succession of transplantations. This similarity of persiste physiology between normal somatic and cancer cells would suggest that t knowledge of the role of heredity in cellular differentiation may be consider here with profit. Wright (1941) has reviewed the subject of heredity relation to differentiation. According to him there are three possible gene hypotheses. The first hypothesis was formulated by August Weismar who postulated a differential sorting out of nuclear determiners in all c lineages except those in direct lineage of actual or potential germ plas This view does not appear tenable in view of the equational character mitosis. Another hypothesis is that certain genes are transmitted in soma divisions in an inactivated condition and that irreversible activation ondarily induced systematically under special local conditions. The ult would be a developing pattern of heredities related to the spatial anization of the embryo. The third and the most probable view is that ular differentiation is fundamentally cytoplasmic in nature and therefore st persist and be transmitted to daughter cells by cytoplasmic transmission. e objection to this third hypothesis is that it ascribes enormous importance cell lineages to a process that is only rarely responsible for differences ween germ cells, at least within a species. It may be, however, that the re or less complete early isolation of the germ line of higher organisms s come about in evolution by setting apart a line of cells with plasmagenes other similar cytoplasmic components lacking in prosthetic groups and nce deficient in any specialized activity. These cytoplasmic entities may capable, in somatic cells, of combining with such groups emanating from e nucleus to form molecules that multiply thereafter as plasmagenes of a ore specialized sort. Under this viewpoint, differences in the local conditins of morphogenesis may bring about differential accumulation of metalic products arising from the interaction of cytoplasmic entities with clear products and may eventually lead to the elaboration of new speciald plasmagenes in the cytoplasm of cells in particular regions of the organn. As development proceeds, each step in the regional differentiation of toplasmic heredity increases the diversity of cellular types and thus facilites further differentiation.

MECHANISM OF THE ACTION OF CARCINOGENS

A brief review is given below of the attempts made in different directions understand this mechanism.

Larionow (1941) investigated the general systemic effects of carcinogenic drocarbons applied to the skin of mice by determining the ratios of parally oxidized substances excreted in the urine, the oxidation-reduction tentials of the blood, and the oxygen consumption of slices of organs. he local effects of carcinogens upon oxidation processes were studied in ssue slices and in skin to which the hydrocarbons had been applied. The idation processes were not disturbed during the precancerous period of apilloma formation but they supervened in a secondary manner after the ppearance of carcinomas. Incomplete investigations of proteins of induced mors indicated that they contained an abnormally large proportion of -glutamic acid. Traumatization of the nervous system by section of the iatic nerve and treatment of the central end with formalin and croton oil ffected the incidence of induced carcinoma in mice. Notic (1942) has conrmed the role of the nervous system.

Berenblum (1941) studied the effect of croton resin on carcinogenesis under arying conditions. The augmentation of carcinogenesis was very proounced when croton resin was applied to the skin concurrently with a dilute olution of a potent carcinogen (3,4-benzpyrene). No effect was observed ith concentrated solutions of carcinogens (3,4-benzpyrene: 1,2,5,6-dibenznthracene; 1,2-benzanthracene), irrespective of their potency. Treatment f the mouse skin with croton resin for a period of 26 weeks failed to influence the response to the subsequent application of benzpyrene. On the otl hand, croton resin applied to the skin subsequent to a limited period l benzpyrene treatment led to a striking increase in the development of tumo Croton resin applied to papillomas already established appeared to bri about their earlier conversion into malignancy.

Jones (1942a, 1942b, 1942c) studied the distribution of carcinogenic hyd carbons and their metabolites by spectographic analysis after a subcutaneo injection of 1,2,5,6-dibenzanthracene in olive oil. Of 45 gm. of hydrocarb injected in 32 rats (total 1000 gm.), 231 mg. could be detected in the excre in an unchanged form, and 43 mg. as 4′-8′-dihydroxy-1,2,5,6-dibenzanth cene. Jones also showed that approximately 36 per cent of the hydrocarbo injected was stored in vesicles formed near the site of injection. No eviden of the presence of phenolic metabolites detected in the excreta was found any of the several tissues examined. A group of workers at The Memor Center for Cancer and Allied Diseases, New York, N. Y. (Dobriner *et a* 1942; Stevenson *et al.*, 1942) did a large amount of work in the study metabolites found in the excreta of injected animals. A large amount of t injected carcinogen is still unaccounted for by any investigator.

Systematic attacks on the problem of the action of carcinogens have bee undertaken by two groups of workers from entirely different angles. Th group of workers at Pasteur Institute, Paris, France, under the direction Lacassagne (1948) has concentrated its attention on the structure of th molecule responsible for its carcinogenic action. The group of workers at th Barnard Free Skin and Cancer Hospital, St. Louis, Mo., under Cowdr (1948), has concentrated on the study of biochemical changes that take pla in the epidermis of the skin of mice following the application of a carcinoge

Workers at the Barnard Hospital have measured several properties skin epidermis, both before and after the application of methylcholanthren The first biochemical alteration appears, with a few exceptions, in the fir five days of methylcholanthrene treatment and persists to the time of th appearance of cancer. A new kind of equilibrium is established in man chemical properties of the hyperplastic epidermis. The changes noted a in some respects different in hyperplastic precancerous epidermis and cancer. In cancer the decrease in calcium ratio is marked (75 per cent The tumorous tissue shows an inability to take in calcium. The nondiffus ble calcium is probably bound to protein. Many other changes have bee noted in both (precancerous and cancerous) conditions. As a workin hypothesis, the St. Louis group has considered the idea that malignant chan is a somatic mutation occurring in a small number of cells, falling in li with the definition of somatic mutation of Strong (1926).

The investigations in Paris were stimulated in 1939 by O. Schmidt hypothesis that the carcinogenic hydrocarbons are characterized by a hig density of mobile electrons in the several so-called activated regions. Th average density of these electrons in these regions would become higher an higher with the increasing number of benzene rings and with the number an nature of substituted groups until a maximal number would be reached. B the use of an empirical technique for estimating electronic density, Schmi e to the conclusion that the mesopositions of the anthracene structure the regions with high electronic density. He thinks that in a carcinogenic rocarbon the electronic density of the active part must exceed a certain ue. This he calls a critical density, and evaluates it as 0.44 π-electrons angstrom square. He also explains the influence of various substituents; example, the methyl group increases the density of the active zone if ced near it, because these compounds help to compress the π-electrons, ereas nitrogen causes the reverse. Schmidt thinks that a carcinogenic rocarbon catalyzes a particular chemical reaction in the cell and therefore s necessary that the part rich in electrons should reach certain cellular mations. Finally, he explains the effect of radiation in parallel with mical substances, giving a unity to his hypothesis. Several of these oretical considerations are supported by the experimental findings of the up of Paris workers (Daudel and Daudel, 1948; Pullman and Pullman, 8). By more refined techniques they have shown that a minimal elec- nic concentration of 1.29^e is necessary for the appearance of carcinogenic ivity and that above this value the degree of activity is proportional to excess of concentration.

n order to visualize a possible relationship between the genetic nature of spontaneous, the transplanted, and the induced tumors, together with chemical nature of the carcinogenic compounds, the production of ger- nal mutations in animals, and the theoretical implications involved in h a complex set of biological phenomena, Strong (1949) has developed the lowing concept: "The carcinogenic hydrocarbons have at least two k ions at which electronic vibrations are maximal. It has been suggested t it is perhaps at these k regions, if not at other regions, that the carcino- ns or some of their metabolites are capable of combining with a nucleo- otein of the cell. [E.] Boyland has suggested that the above combina- n of a gene with a foreign molecule would interfere with cell division thus ding to chromosomal aberrations. However, all data obtained with ethylcholanthrene on mutagenesis in mice indicate that we are dealing t with chromosomal aberrations but with point mutations. A further nsideration of Boyland's concept is thus indicated. After a carcinogen or e of its metabolites has combined with a unit of nucleoprotein within the ll (that is a gene), it can only be eliminated by oxidation, by demethylation by some other chemical means by which energy is involved. If this ergy be released or absorbed at the electronic level, it could not be dis- pated in the form of heat. Thus a source of available energy at close oximity to a gene may very well combine with such a gene or nucleoprotein d change the energy content of a derived nucleoprotein. The derived cleoprotein would thus be a mutant (a derived or altered biochemical it)."

Summary and Conclusions

(1) The present investigation includes observations on 2966 mice of the Br subline of the NHO strain of mice produced by a single mating of a JK a CBAN in the year 1937. These mice belong to the first 20 generations

of inbreeding. The first 3 generations consisted of 9 mice that were used breeding purposes only. From the fourth generation onward each mou between 60 and 70 days of age, received in its right flank a subcutane injection of 1 mg. of 20-methylcholanthrene dissolved in 0.1 cc. of sesame Mice were either killed when a definite tumor was observed or were allow to live the full life span. A constant attempt to increase the latent periods tumors in mice of succeeding generations was made by selecting breed with increasingly longer latent periods. A total of 2544 tumors (counti all tumors at a single site as 1) was detected at various sites in 2231 mi between the F_4 and F_{20} generations; 1528 pathologic lesions were saved a studied for histology; 561 mice died without revealing any tumor.

(2) A statistical method is devised to study the multivariate effects carcinogen and selection. The variables examined are the anatomical sit histological types, sexes, latent period, and generations. The mice a divided into 3 periods of successive generations, consisting of 580 mice F_4 to F_{12}, 927 mice in F_{13} to F_{16}, and 1285 mice in F_{17} to F_{20}. This procedu leaves adequate numbers of mice in the 3 periods to study the changes the variables concerned. In F_{17} to F_{20} there were 2 families (one with 4 mice and the other with 283 mice), which had a common descent up to th eighth generation and separated thereafter. These 2 families were analyze for anatomical sites, litter seriation, and latent period of local tumors. Th method adopted for the entire analysis makes possible the visualization the effect of carcinogen and selection in terms of probability on either th mouse, the anatomical site, or the histological type of tumor.

(3) A single injection of carcinogen gave rise to tumors of a variety histological types at the site of injection and at other sites. These oth sites include the surface of the body, the lung, forestomach, liver, ovar uterus, and some others. This finding suggsts that the carcinogen has widespread systemic effect.

There are considerable differences in the mean values of latent periods f tumors at different sites: at the site of injection the mean value is 201 day for surface tumors it is 447 days; for the tumors of the forestomach 478 day for lung tumors, 510 days; and for liver tumors, 570 days. There are min variations in the mean values of latent periods of different histological typ at various sites. For local tumors the mean value for epidermoid carcino is 151 days; for fibrosarcoma it is 167 days. There are also differences in th sexes. For lung tumors the mean value is 460 days for females and 532 da for males. Probably there is no effect of selection in lengthening the late period of any specific type of tumor. These findings suggest that tiss susceptibility, as reflected by its latent period, shows considerable differenc under the conditions of the present experiment. However, these differenc are probably not subject to variations under the effect of selection.

There are considerable variations in the incidence of tumors at differe anatomical sites and histological types during F_4 to F_{20}. There are al considerable differences in the incidence of tumors in the sexes. The on major variation in the tumor incidence in males during the experiment between F_4 to F_{12} and F_{13} to F_{16}, reflected in a large decrease (from 56.4 p t to 39.3 per cent) in the incidence of local tumors. The incidence of ological types of local tumors in males does not show much variation. tumor incidence in females shows considerable variations, both at dif- nt anatomical sites and in histological types during F_4 to F_{20}. The inci- ce of liver tumors also shows striking differences in the sexes. This ld suggest that tissue susceptibility could be visualized only in terms of bability expressed here as percentage incidence. Variations in tissue sus- tibility are probably the result of selection. Differences between the es suggest the action of some specific physiological avenues. In contrast these variations in tissue susceptibilities during F_4 to F_{20}, the variations total tissue susceptibility (as measured by the proportion of mice that eloped tumors at any site to all the mice) are not great. This would sug- t that the control of tissue susceptibility might be at a different level in mechanism of heredity than is total tumor susceptibility.

(4) A discussion is included of our knowledge of the genetics of cancer in ce at three different levels; namely, the spontaneous tumor, the trans- nted tumor, and the induced tumor. A probable interrelationship tween them is suggested.

(5) A discussion of the action of carcinogens is also included.

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HEMICALLY INDUCED CARCINOMAS OF THE MAMMARY GLAND AND FIBROSARCOMAS WITH AND WITHOUT SELECTION*

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ata have been published dealing with the influence of a parental-aging or on susceptibility to chemically induced tumors in mice.[1-14] It is clear t there is a progressively changing influence on cancer susceptibility in offspring as the parents age. This changing factor or factors has been wn to be cumulative over a number of generations.[13]

ne of the limiting factors in an experiment of this nature is the relatively ll number of animals that can be obtained in advanced litters of breeding e. In order to correct for this restrictive influence on the analysis of the a on parental age or litter seriation, the following experiment was initiated. the past, no attempt had been made to measure the effect of selection n this changing aging factor that influences cancer in the offspring. No tter how small a variation is, if it be cumulative over a number of genera- ns, then selection should be able to increase or to decrease it. In the sent investigation, selection toward a descent from first and second litters y has been continued for more than ten generations, that is, between –F_{30}. The degree of inbreeding reached, the F_{20}, before selection was d, should have been sufficient to produce a high degree of homozygosity. brother-sister matings, the percentage of homozygosity should have ched 99 per cent in the twentieth generation.

Three successive series of the original pBr descent before selection was orted to had demonstrated that variability of tumor types had become iform (unpublished data). Even a spontaneous mutation rate of one ible mutation in 26,000 mice should not greatly disturb the genetic purity the mice at the time selection was resorted to, nor even during the experi- ent. The percentage of invisible mutations, such as lethals and semi- hals, is still an unknown factor in mice. For the time being, it remains corrected, but must be of slight influence.

There are two purposes in presenting the present data. These are briefly scussed above and are as follows:

(1) The addition of further data obtained on the incidence of chemically duced tumors in the 2-Prunt descent, particularly of mice belonging to vanced litters.

(2) The introduction of selection toward an early litter descent for at ast ten generations. This procedure of selection is the same as restricting e potential size of the family over a number of generations.

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Due to unforeseen complications, neither of the two purposes for which t present experiment was undertaken could be completely realized. Th will be discussed later.

Materials and Methods

All mice used in this experiment belonged to the 2-Prunt descent, t origin of which has been described previously. Briefly stated, the mice the 2-Prunt descent were separated off from the pBr descent in the 1 generation. All mice of the pBr descent had been injected with 20-meth cholanthrene between the F_4–F_{20}. Following the separation of the 2-Pru descent in the F_{20} generation, breeding mice no longer were injected, that t parents received no methylcholanthrene; only those offspring who were n used to carry on the 2-Prunt line of descent were injected with the carcinog

The present experiment deals with two series of the 2-Prunt descent. Th first series consists of the descendants of mice of the original 2-Prunt desce beginning with F_{21}. These mice have been continued without selectio that is, the 2-Prunt parents were continued as breeders as long as they wou breed. Their offspring of any generation that were used for breeders of t next generation were mated together irrespective of litter seriation or t age of mothers when they were born. The second series of 2-Prunt mice an independent line of descent from the same mice of the F_{20} generati that have been restricted toward an early litter descent. The second seri started with mice of the F_{30} generation. In the present paper, therefore, t relative incidence of the various types of chemically induced tumors a recorded for mice of these two descents. Mice of the second series we treated the same as those mice of the first series as far as the injection of t carcinogen is concerned, but have been selected by an early litter descent f at least 10 generations. Mice of the second series, therefore, were betwe the F_{30}–F_{40} generations of inbreeding. By this method it is hoped to analy the effect of selection toward a suppression of the total size of the famil beginning with a relatively homozygous condition, on susceptibility chemically induced tumors.

All mice were weaned at approximately 4 weeks of age, placed in reserv except those set aside for breeding purposes as noted above and, at 60 da of age, injected in the right flank with 1 mg. of 20-methylcholanthrene d solved in 0.1 cc. of sesame oil. The sexes were kept separated and the anima were inspected for tumors weekly, beginning approximately at the seven week after the injection of the carcinogen. The latent period was determin as the time between the injection of the carcinogen and the appearance o definitely growing tumor as determined by palpation. The mice were ke alive until death from the growth of the tumor or from other diseases occurre The survival time was taken as the time between the first gross appearan of the tumor and the death of the animal. Occasionally a tumor-beari mouse was sacrificed when *in extremis* or when it showed signs of sickne such as loss of weight, wheezing, ruffled hair, and laborious breathing. pathological lesions were fixed in Bouin's solution and prepared and stain by hematoxylin and eosin for histological examination.

Observations and Data

e animals used in this study consisted of 800 female and 632 male mice e 2-Prunt subline. The chemically-induced tumors in both the females males were studied initially and then a more detailed study of the tumors e females was undertaken. A total of 1843 pathological specimens was ined and diagnoses were made, wherever possible. The tumors were equently classified into 9 categories. Of the 1843 pathological specimens, were from female mice, of which 1040 were diagnosed; 702 were from mice, of which 654 were diagnosed. Some of the tumors could not be nosed due to post-mortem changes.

cal tumors (those arising at the site of the injection of the carcinogen) divided into 4 groups: (1) squamous cell carcinoma, (2) round cell oma, (3) fibrosarcoma, and (4) mammary gland carcinoma. The mam- y gland carcinomas and all squamous cell carcinomas of the skin were ally restricted to female mice, but in the later groups of mice there were les with 5 mammary gland carcinomas, as well as 1 male with an epider- l carcinoma. Not only the tumors that arose at the site of the injection ethylcholanthrene, but also any tumors that appeared on the surface of trunk or neck were grouped together as local tumors, since these "surface ad" tumors[3] were mostly mammary gland carcinomas. Some few fibro- omas in both males and females appeared at sites other than those at h the methylcholanthrene had been injected (right flank). In mice, normal mammary tissue covers nearly three fifths of the body ace.

he categories other than local (including surface spread) consisted of r tumors, lung tumors, others (of rare occurrence), and collision tumors, vell as unidentified sections. The "others" category included all those t were identified, but were not local, lung, liver, or collision tumors. lision tumors were those, local or otherwise, that showed two or more plastic elements within the same section (for example, sarcoma and cinoma, or squamous cell carcinoma and adenocarcinoma). The unidenti- category included all those sections for which the diagnosis was seriously stionable or that were completely unidentifiable due to post-mortem nges. Some of these post-mortem changes were due to the fact that mice h tumors would die during the night and could not be autopsied until the t day. In addition, any section that was not neoplastic was included this unidentified category. The mammary gland carcinoma group braced all of the various types of mammary gland carcinomas, including a epidermoid carcinomas that appeared to originate definitely from mam- ry gland tissue. Most of the mammary gland tumors, however, were ly well differentiated adenocarcinomas.

The two types of tumors that were more thoroughly analyzed were fibro- coma and mammary gland carcinoma, since these two were of most fre- ent occurrence. Tumors were classified as fibrosarcoma whenever any ndle cells were observed, even though the section may have consisted gely of round cell elements. The mammary gland carcinomas were largely

TABLE 1
2-PRUNT FEMALE MICE

		Animals Nos. 1–100		Animals Nos. 101–200	
		Total No. of tumors: 123		Total No. of tumors: 1	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	52.85	141.1	52.90	130.5
	Squamous cell CA	13.82	113.9	9.42	94.0
	Mammary gland CA	9.76	148.8	8.70	148.3
	Round cell sarcoma	3.25	163.0	7.25	121.3
Lung		4.88	120.7	7.97	143.1
Liver		2.44	216.3	2.17	112.1
Others		5.69	147.6	3.62	103.6
Collision		7.32	140.0	7.97	153.3
Unidentified sections		4		6	

		Animals Nos. 201–300		Animals Nos. 301–400	
		Total No. of tumors: 121		Total No. of tumors: 12	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	54.55	169.6	57.72	123.2
	Squamous cell CA	4.13	139.0	4.88	77.7
	Mammary gland CA	20.66	164.7	25.20	118.3
	Round cell sarcoma	—	—	—	—
Lung		9.09	222.8	6.50	160.0
Liver		1.65	152.0	0.81	87.0
Others		1.65	154.0	—	—
Collision		8.26	132.5	4.88	100.0
Unidentified sections		12		9	

adenocarcinomas, with a few showing variations in the medullary a scirrhous directions.

The mice were divided into groups of 100 according to their chronologi order of death and according to sex. Thus, the first 100 female mice to were numbers 1–100, the second 100 females to die were numbers 101–2 etc. The same held true for the male mice. TABLE 1 shows the percenta incidence and average latent periods for all 8 groups of the females. TABL shows the same information for all the males (7 groups). TABLE 3 gives t

TABLE 1 (*Continued*)

		Animals Nos. 401–500		Animals Nos. 501–600	
		Total No. of tumors: 127		Total No. of tumors: 133	
		Percentage incidence	Latent period	Percentage incidence	Latent period
ul	Fibrosarcoma	48.03	111.8	37.60	139.3
	Squamous cell CA	6.30	98.8	10.53	149.1
	Mammary gland CA	22.83	130.6	34.59	118.9
	Round cell sarcoma	3.94	102.6	0.75	138.0
g		13.39	172.2	9.77	204.8
r		—	—	—	—
ers		1.58	79.0	—	—
ision		3.94	130.0	6.77	111.4
dentified sections		13		24	

		Animals Nos. 601–700		Animals Nos. 701–800	
		Total No. of tumors: 135		Total No. of tumors: 140	
		Percentage incidence	Latent period	Percentage incidence	Latent period
cal	Fibrosarcoma	39.26	135.0	40.71	178.0
	Squamous cell CA	5.19	177.6	5.00	96.9
	Mammary gland CA	34.07	137.2	37.86	176.1
	Round cell sarcoma	1.48	144.0	0.71	120.0
ng		12.59	211.2	10.00	357.1
er		0.74	136.0	0.71	107.0
hers		2.96	223.0	—	—
llision		3.70	139.6	5.00	111.0
identified sections		17		14	

Data on the percentage incidence of the various types of tumors encountered in female Prunt mice only, together with the average latent period for each type. The data are parated into eight groups of 100 mice each, based upon the chronological order of death the mice.

ormation for the entire group of females and males without division into oups of 100 (the latent period is the length of time from the injection of ethylcholanthrene to the first gross appearance of a definite tumor).

One of the most conspicuous features of the data in male mice is the occurnce of only a single squamous cell carcinoma. Noteworthy too, is the currence of 5 mammary gland tumors in 3 male mice. Also, when com-

TABLE 2
2-PRUNT MALE MICE

		Animals Nos. 1–100		Animals Nos. 101–200	
		Total No. of tumors: 104		Total No. of tumors: 10	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	82.69	167.5	85.15	167.4
	Squamous cell CA	—	—	—	—
	Mammary gland CA	—	—	—	—
	Round cell sarcoma	2.89	203.3	4.95	423.4
Lung		8.65	279.6	3.96	330.5
Liver		—	—	0.99	506.0
Others		5.77	208.0	2.97	428.7
Collision		—	—	1.98	108.0
Unidentified sections		10		5	

		Animals Nos. 201–300		Animals Nos. 301–400	
		Total No. of tumors: 101		Total No. of tumors: 10	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	87.13	181.8	82.08	179.4
	Squamous cell CA	—	—	—	—
	Mammary gland CA	—	—	—	—
	Round cell sarcoma	—	—	1.89	142.0
Lung		10.89	463.0	8.49	372.9
Liver		0.99	148.0	4.72	338.6
Others		0.99	157.0	2.83	446.7
Collision		—	—	—	—
Unidentified sections		10		9	

pared with the data in female mice, males showed a somewhat higher in dence of local tumors (84.10 per cent for the males, 81.72 per cent for t females).

The data in TABLE 1 show that there is an increase in the incidence mammary gland carcinoma, rising from 9.76 per cent in the first gro (1–100) to 37.86 per cent in the last group (701–800).

The complete group of female mice (800) was further divided into t different series. The first series includes most of the first 300 animals p

TABLE 2 (*Continued*)

		Animals Nos. 401–500		Animals Nos. 501–600	
		Total No. of tumors: 106		Total No. of tumors: 103	
		Percentage incidence	Latent period	Percentage incidence	Latent period
l	Fibrosarcoma	80.19	178.5	70.87	196.1
	Squamous cell CA	—	—	0.97	141.0
	Mammary gland CA	2.83	317.0	0.97	262.0
	Round cell sarcoma	2.83	125.7	4.85	244.0
g		12.26	408.2	21.36	472.6
r		—	—	—	—
ers		—	—	—	—
ision		1.89	283.0	0.97	136.0
lentified sections		4		8	

		Animals Nos. 601–632	
		Total No. of tumors: 33	
		Percentage incidence	Latent period
Local	Fibrosarcoma	63.64	186.9
	Squamous cell CA	—	—
	Mammary gland CA	3.03	97.0
	Round cell sarcoma	—	—
Lung		30.30	466.2
Liver		3.03	162.0
Others		—	—
Collision		—	—
Unidentified sections		2	

Data on the percentage incidence of the various types of tumors encountered in male runt mice, together with the average latent period for each type. The data are separated into seven groups based upon chronological sequence of death.

usly classified in TABLE 1, and the other series includes the remainder. e first series comprises females whose parents were unselected with respect litter seriation. The females in the second series were the offspring of rents born in early litters (first and second litters only).

The first series consists of 308 female mice and the second series contains 2 female mice. TABLE 4 shows the percentage incidence of the various

TABLE 3
2-PRUNT MICE

		Males, 632 Animals		Females, 800 Animals	
		Total No. of tumors: 654		Total No. of tumors: 104	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	80.43	178.4	47.69	140.6
	Squamous cell CA	0.15	141.0	7.40	118.4
	Mammary gland CA	0.77	262.0	24.42	142.7
	Round cell sarcoma	2.75	256.0	2.21	127.1
Lung		11.93	418.6	9.33	208.3
Liver		1.22	313.6	1.06	147.3
Others		1.99	310.1	1.92	145.5
Collision		0.77	183.6	5.96	129.0
Unidentified sections		48		101	

Data on the whole male and female groups. The percentage incidence and avera latent period are given for the various tumor categories.

TABLE 4

		Series I, 308 Animals ♀		Series II, 492 Animals ♀	
		Total tumors: 388 1.26 tumors per mouse		Total tumors: 652 1.33 tumors per mouse	
		Percentage incidence	Latent period	Percentage incidence	Latent period
Local	Fibrosarcoma	53.09	150.2	44.48	134.6
	Squamous cell CA	9.02	120.1	6.44	117.0
	Mammary gland CA	12.63	162.4	31.44	137.9
	Round cell sarcoma	3.61	133.2	1.38	117.7
Lung		8.25	216.4	9.97	204.1
Liver		2.06	161.3	0.46	110.0
Others		3.61	132.8	0.92	175.0
Collision		7.73	141.6	4.91	117.2
Unidentified sections		27		74	

The same data as in TABLE 1 without a separation according to chronological order death; instead, the mice are separated into the two series.

or types and the average latent periods for these two series of mice. inspection of the data in TABLE 4 reveals that there are definite differ- es between the two series. The incidence of mammary gland carcinoma s from 12.63 per cent in the first series to 31.44 per cent in the second es. This rise is accompanied by a slight decrease in percentage incidence he other tumor types (except for lung tumors). The average latent iod for all the tumor groups except "others," shows a definite decrease in second series. There is a slight insignificant increase in the number of ors per mouse, from 1.26 in the first series to 1.33 in the second series.

he frequency of single and multiple tumors of the fibrosarcoma and mam- ry gland carcinoma types was determined. A multiple tumor is defined

TABLE 5

	Single		Multiple		Total	
	No.	%	No.	%	No.	%
SERIES I						
rosarcoma	155	75.24	51	24.76	206	100.0
mmary gland CA	27	55.10	22	44.90	49	100.0
SERIES II						
rosarcoma	206	71.03	84	28.97	290	100.0
ammary gland CA	69	33.66	136	66.34	205	100.0

The percentage incidence of single and multiple tumors in female mice, within the fibro- coma and mammary gland carcinoma groups. Data for the two series are listed parately.

re as one that has one or more other tumors present in the same animal gardless of the pathological type. TABLE 5 presents these results. There no significant change in the fibrosarcoma group in the two series, but the ammary gland carcinoma group shows an increase in multiple tumors, from .90 per cent in the first series to 66.34 per cent in the second series.

Each of the two series of female mice was studied further to note any cor- lation between birth rank of the tumorous animals and the onset of fibro- rcoma or mammary gland carcinoma. The latent period and survival me for the animals in each litter rank were plotted against litter number. IGURE 1 shows these data for the first series. Latent period and survival me were plotted on the ordinate in days and the litter number is expressed n the abscissa. Survival time represents the period from the first appear- nce of the tumor until the death of the animal. In the first series, female ice developed fibrosarcoma on an average of 150.2 days irrespective of litter eriation; they developed adenocarcinomas of the mammary gland on an average of 162.4 days. Survival times for the same mice were 76.9 days fo fibrosarcoma, and 103.0 days for female mice bearing adenocarcinomas the mammary gland. Thus, the latent period and survival time for mammary gland carcinomas are both longer than similar data for fibrosarcoma (162.4 days compared to 150.2 days, and 103.0 days to 76.9 days). Ther appears to be a slight decrease in survival time of mice in later litters for bot fibrosarcoma and mammary gland carcinoma.

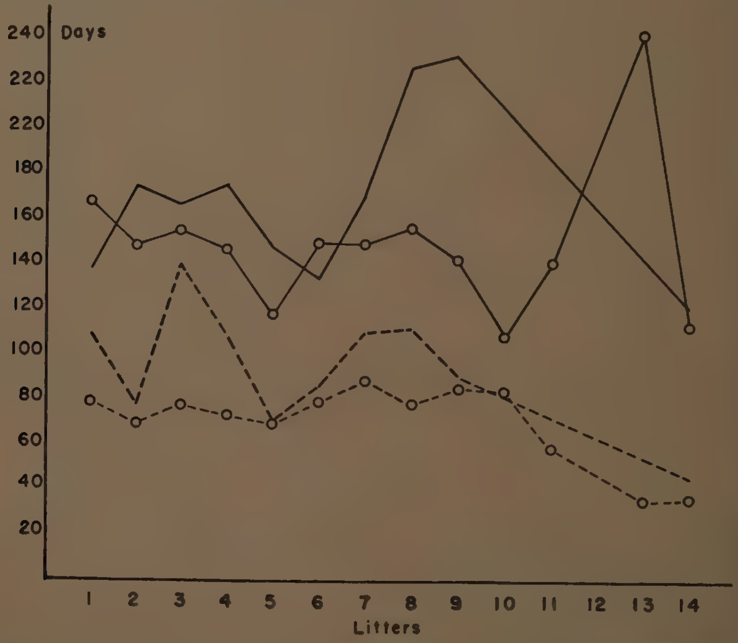


FIGURE 1. Data on characteristics of the latent period and survival time in relation t birth rank for mammary gland carcinoma and fibrosarcoma of the first series 2-Prun females are shown. Latent period and survival time in days are shown on the vertica line and litter number or rank on the horizontal line. For fibrosarcoma the latent period i on the solid and open circle line, the survival time on the short dash and open circle line; fo mammary gland carcinoma, latent period is on the solid line, survival time on the sho dash line.

FIGURE 2 shows corresponding data for female mice of the second series Here, the average latent periods of fibrosarcoma and mammary gland carcinoma are, unlike the first series, approximately of the same value (129. and 131.4 days, respectively). The survival time of mice bearing eithe type of tumor is also very similar; with mammary gland carcinoma agai slightly but not significantly higher than fibrosarcoma (92.5 compared t 89.1 days, respectively). No consistent differences are manifest accordin to litters.

Comparative data for the two series of 2-Prunt female mice are shown i FIGURES 3, 4, 5, and 6. FIGURES 3 and 5 compare the frequency of fibro oma and mammary gland carcinoma, relative to one another, in the two es, according to litter seriation. An increase of mammary gland carcinoma he intermediate litters over that for fibrosarcoma occurs in the second es. Here (FIGURE 5) the percentage of mammary gland carcinoma starts , increases in the intermediate litters, and then falls again. The tenth er is not significant, since it represents but one mammary gland tumor and ibrosarcoma. In the first series a decrease in mammary gland carcinoma urs in the later litters (FIGURE 3), but these litters (10–13) comprise only

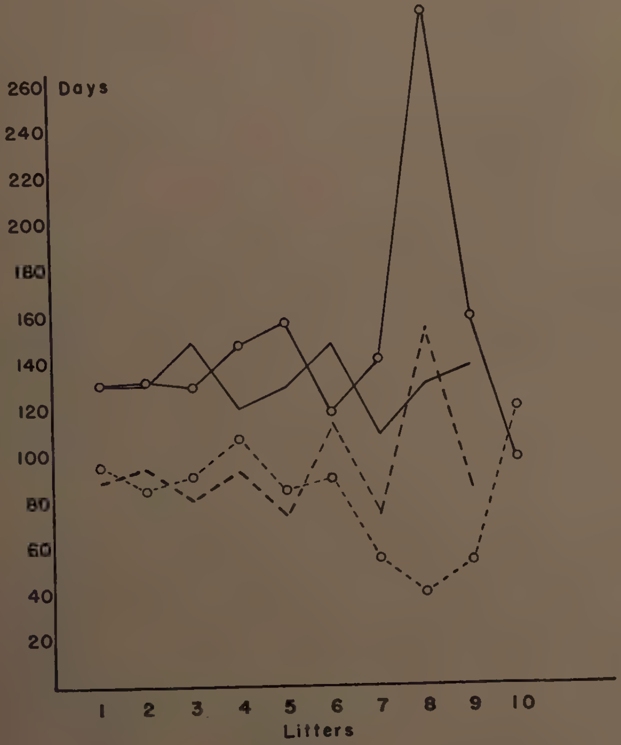


FIGURE 2. Data comparable to that in FIGURE 1 are shown for the second series of the Prunt female mice.

very few animals with no mammary gland carcinoma, and therefore are ot significant. Essentially the same things are shown by 3-point moving lots (FIGURES 4 and 6).

The characteristics of the latent period and survival time of one series as ompared to those of the other series for each tumor type (mammary gland arcinoma and fibrosarcoma) are graphically represented in FIGURES 7 and 8. comparison of the fibrosarcoma group (FIGURE 7) shows a definite differ- nce between the latent periods of the first and second series. The latent eriod in the second series is less than that in the first series in all but one tter (litter No. 5). The average for the entire group of series No. 2 (134.6)

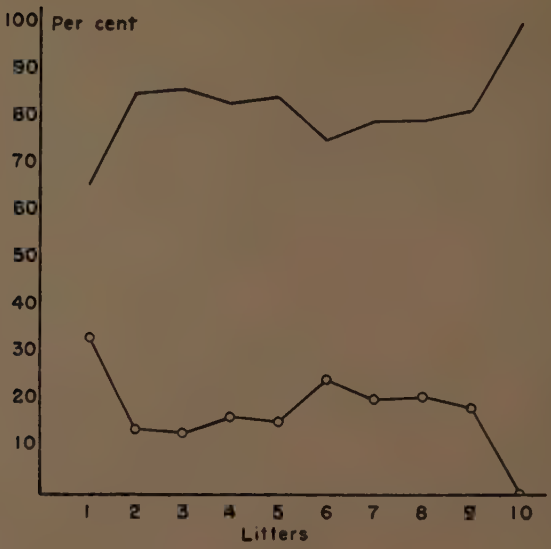


FIGURE 3. Percentage incidences of fibrosarcoma and mammary gland carcinoma rel tive to one another for female mice of the first series. Fibrosarcoma data are on the soli line, mammary gland carcinoma on the solid and open circle line. Only data for the fir ten litters are included. Beyond the tenth litter the data are too meager for inclusion.

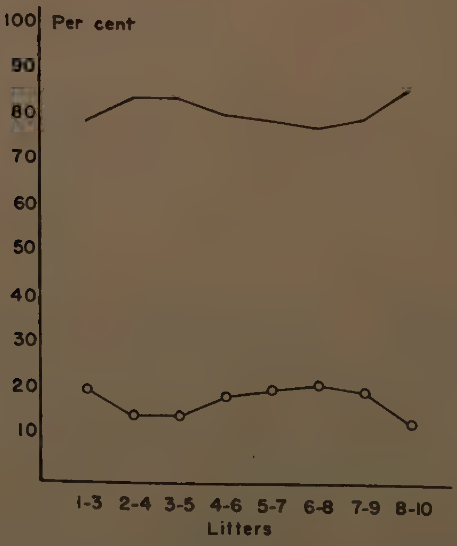
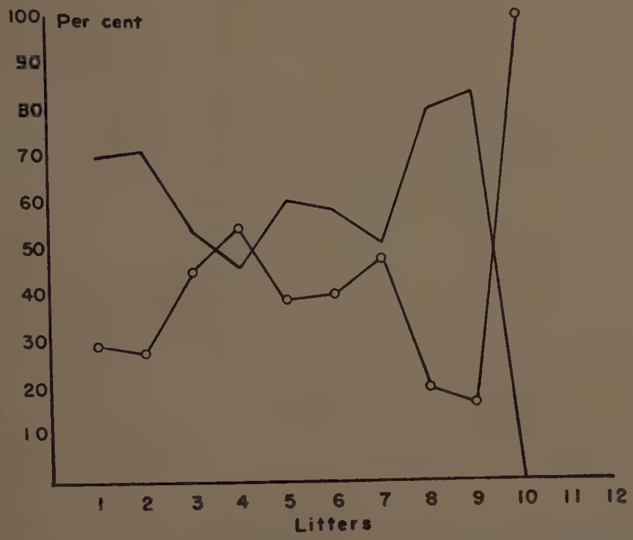


FIGURE 4. Three-point moving curve of data in FIGURE 3.



IGURE 5. Percentage incidences of fibrosarcoma and mammary gland carcinoma rela- to one another for female mice of the second series. Fibrosarcoma data are on the d line, mammary gland carcinoma on the solid and open circle line.

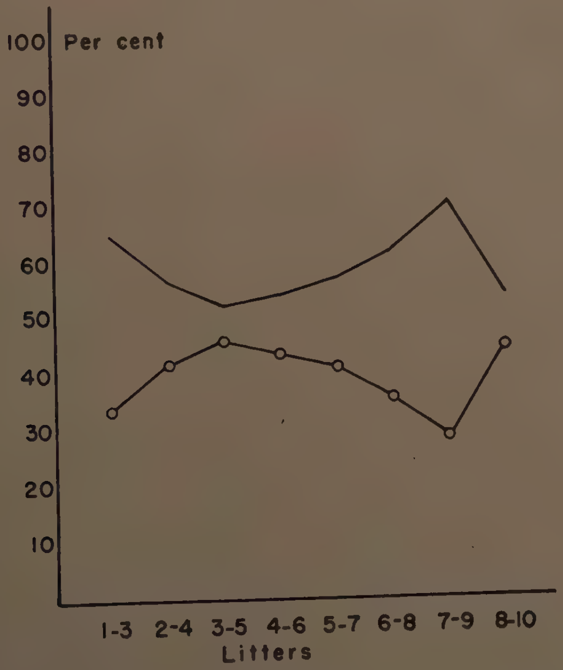


FIGURE 6. Three-point moving curve of data in FIGURE 5.

is 15.6 days less than that of series No. 1 (150.2). There is an increase i the latent period of the first series in the last few litters, which is probabl not significant due to the small number of animals in these litters. Th survival time of mice of the first series is slightly below that of the secon series in all but one litter (No. 7), and its average (76.9) days is 13.5 days le than that of the second series (90.4 days). Summation of the average laten period and average survival time data for each series shows the average lif span[1] of the first series (225.0 days). Thus, fibrosarcomas came earlie in the second series of female mice, but the mice had also longer survival times

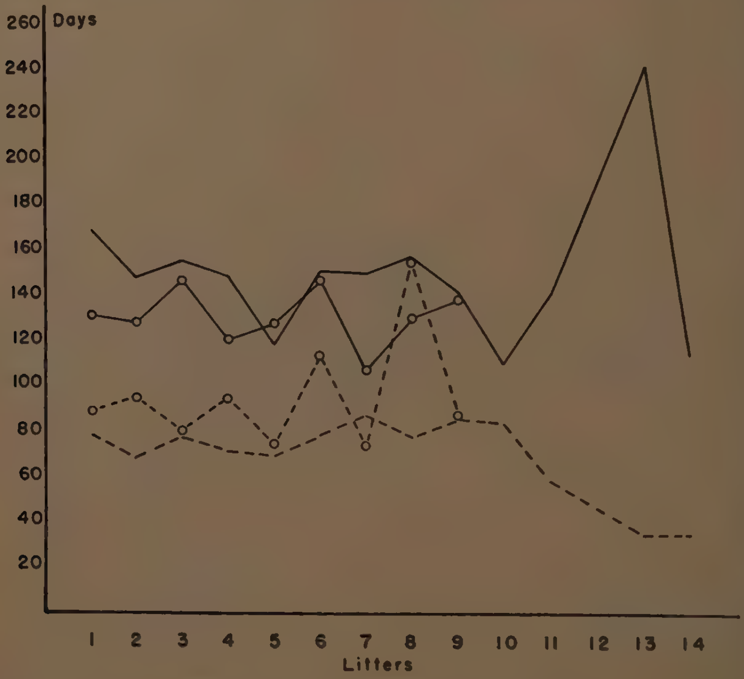


FIGURE 7. Data for latent period and survival time for female mice bearing fibrosarcoma of both the first and second series. For the first series the latent period data are o the solid line, survival time data on the short dash line. In the second series, data fo the latent period are on the solid and open circle line, survival time on the short dash an open circle line.

An examination of the mammary gland carcinoma groups of the first an second series (FIGURE 8) shows that the average latent period and the surviva time of the first series are greater than those of the second series (162.4 day compared to 137.9 days for the latent periods, and 103.0 days compared t 92.2 days for the survival times, respectively). The latent period of th first series is longer than that of the second series with the exception of litter No. 5 and No. 8. The latent periods of both series show some rise in th later litters, but this is probably not significant because of the small number

nimals in the later litters. The survival times show no consistent differ-
es between the two series (FIGURE 8).

he sum of the average latent period and average survival time (life span*)
he first series (265.4 days) exceeds that of the second series (230.1 days),
the mammary gland carcinoma group. The difference in life spans
etween mice of the first and second series bearing adenocarcinomas of the
ammary gland is thus 35.3 days.

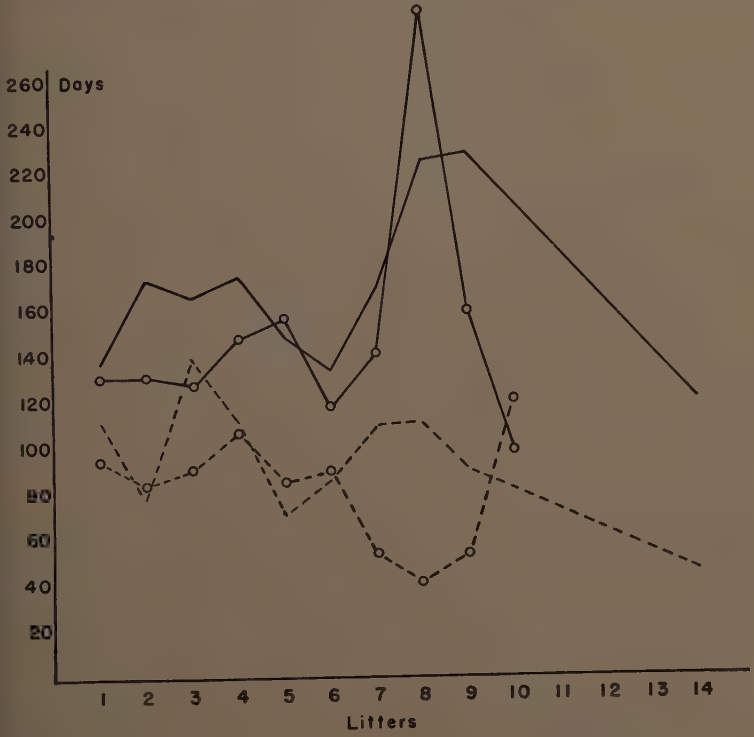


FIGURE 8. Data on the latent period and survival time of female mice of both series
aring adenocarcinoma of mammary gland. Latent period for female mice of the first
ies is on the solid line; survival time for these mice of the first series is on the short dash
e. For data for the mice of the second series, the latent period is indicated on the solid
d open circle line, survival time on the short dash and open circle line.

In the male data of TABLE 2 changes are evident in the lung and fibro-
rcoma groups. The percentage incidence of fibrosarcoma starts at 82.69
er cent in the first 100 male mice, and after a slight rise it gradually decreases
63.64 per cent in the last group. Concomitantly the lung tumors rise
om a low of 3.96 per cent in the second 100 male mice to 30.30 per cent in
e last group. FIGURE 9 shows that at the same time all other tumor types
aintain a fairly constant percentage incidence. The last group (the

*Life span is latent period plus survival time; it does not include the period of time
om birth to injection of the methylcholanthrene, which is about sixty days.

seventh) is not as significant as the others, since it represents only 32 mi In contrast, female mice showed only a relatively slight variation in percen age incidence of lung tumors.

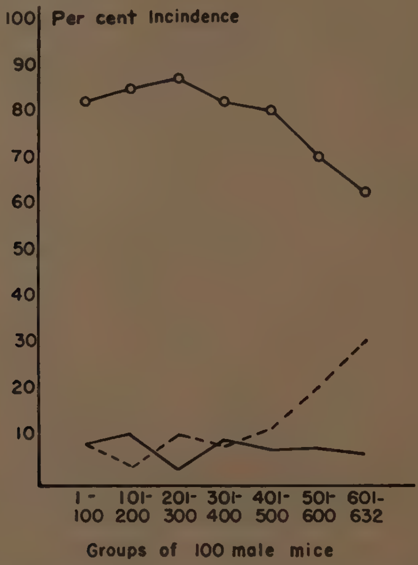


FIGURE 9. Percentage incidence of fibrosarcoma, lung tumors, and all other tumors f male mice. Percentage incidence data are on the ordinate; successive groups of 100 ma mice on the abscissa. Data for fibrosarcoma are on the solid and open circle line, for lu tumors on the short dash line, and for all other tumors on the solid line.

General Discussion

As indicated earlier, unforeseen complications prevented realization of t two purposes of the present experiment. These were (1) the addition of mo data to a similar study already published, particularly of mice belonging advanced litters, and (2) altering susceptibility to chemically induced fibr sarcomas by the use of selection based upon maternal age. It soon becam apparent that mice belonging to advanced litters were not being obtain with selection toward a first and second litter descent, or a selection towa the suppression of the total potential size of the family. Thus it becom impossible to add data to the older data of birth rank trends (litter seriatio when further data would be desirable to increase the possible significance these trends.

Instead of solving a problem, the selection experiment, has merely intr duced another complicating one. Instead of measuring changes in fibr sarcoma susceptibility by selecting toward an early litter descent, in fema mice the tumor is gradually being replaced by the adenocarcinoma of t mammary gland. Only part of this increase in adenocarcinoma of t mammary gland can be explained in a lessened incidence of fibrosarcom The rest must be due to a suppression of all other types of late-appeari

ors. The fact that, with but one exception, the female mouse alone of
selected 2-Prunt descent, and not the male, develops squamous cell
inoma of the skin is striking, but the reason for this is quite unknown.
almost all of these shiftings of tumor incidence and survival times it is
female that is undergoing these biological variations. This same phe-
enon was also encountered in the ancestral stock (the pBr) that has given
to the two series of 2-Prunt descents in the present experiment (Strong
Sanghvi[9]). In one instance, however, there was a shift in fibrosarcoma
lence in the male that was compensated for by an altered incidence of
tumors.

erhaps the two observations that did come out of the present experiment
of as much interest as the two that underlay the formulation of the
eriment.

is still not certain whether these two observations, namely (1) the
ility of mice to produce advanced litters, and (2) the partial suppression
brosarcomas and other late-appearing tumors and the increase of adeno-
inomas of the mammary gland in female mice injected with methyl-
anthrene, brought about by the selection toward the suppression of the
ential size of the family, are permanent to the species or only of a tem-
ary nature. This can be determined only by reversed selection, and it
ld be unwise to do this until biological equilibrium has been established.
iscussion of mechanisms involved should wait.

Conclusions

) The selection toward an early litter descent (first and second litters
) results in a biological state in which the mice are no longer capable of
ducing advanced litters.

2) The same selection brings about the suppression of the chemically
uced fibrosarcoma and other late-appearing tumors (except lung) and an
ease of adenocarcinoma of the mammary gland in female mice.

3) The increase in the incidence of adenocarcinoma of the mammary
nd takes place at the expense of all other types of chemically induced
iors except lung adenomas.

4) Selection appears to increase susceptibility (as measured by latent
iod) to mammary gland carcinoma, but does not appear significantly to
er susceptibility to fibrosarcoma in females of the same strain.

5) An inverse relationship between lung tumors and fibrosarcomas occurs
nale mice, the lung tumors increasing and the local fibrosarcomas decreas-
with continued selection.

6) The appearance of five adenocarcinomas of the mammary gland in
ee male mice may be of significance.

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MATERNAL AGE SELECTION AND CHEMICALLY INDUCED TUMORS IN MICE*

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n recent years there has been an increasing interest in the possible rela-
ship between the aging process and susceptibility to cancer. Studies
e been conducted to determine relationships of heredity and aging to
cer in humans.[3, 27, 28] One disadvantage in attempting to analyze the
blem in human populations with respect to genetics is the high degree
uncontrolled variability of heterozygosity. There have been several
orts linking cancer susceptibility to specific genes in inbred strains of
e.[6, 11, 12, 14, 20] One objection to applying this type of information to the
nan population is that inbred mice have a high degree of genetic homozy-
ty, whereas the human population has a very low degree of genetic
nozygosity. To approximate human conditions better, it would be desir-
e to have a descent of hybrid mice derived from several different highly
red strains varying in susceptibility to cancer.

hybrid descent combining the genetic factors of several inbred strains
nice that varied in susceptibility to chemically induced cancer was pro-
ed by L. C. Strong in 1937. The NH strain was derived from the CBA,
J, and K strains of inbred mice. The differences in response to induced
nors in these strains have been published.[20] In general, there is a low
dence of spontaneous tumors in the ancestral stocks.

escendants of the NH strain were injected with 20-methylcholanthrene
named the NHO strain. This potent carcinogen apparently induced
ne mutations in the NHO strain.[16] It was subsequently found that there
s some relationship between cancer susceptibility and these mutations.[12, 14]

Jsing the NHO strain, a study was conducted to produce a strain rela-
ely resistant to induced tumors by selection of offspring from animals
ving long latent periods, that is, from time of injection of the carcinogen
il the first appearance of the tumor.[13, 18, 19] Results opposite to those
ected were obtained, namely that selection toward resistance to cancer
ceptibility actually eventually *increased* susceptibility as manifested by
rter and shorter latent periods in offspring of resistant animals.

everal inbred strains of descendants of the NHO strain, namely the
unt, 2-Prunt, 4-Prunt and 5-Prunt sublines, the origins of which will be
cussed later, have been used in studies to determine the effect of aging
cancer susceptibility. Maternal age selection or litter seriation has been
d as the variable for aging. Animals selected toward first and second
ers differ from animals selected toward late litters in respect to age of the

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illment of the requirements for a degree of Doctor of Medicine obtained in June 1957.
s based largely on experiments made possible by a grant awarded to Leonell C. Strong
The National Cancer Institute, Public Health Service, Bethesda, Md.

mother at the time the offspring were born. That is, offspring from ear litters were born while their mothers were young, on the average, while o spring from late litters were born when their mothers were older on t average. If there is some factor or factors related to the aging process whi affects cancer susceptibility, its effects can be determined by compari animals selected toward early litters with animals selected toward late litte Data already published indicate that there is an influence on cancer su ceptibility in the offspring as parents age. The effects of this influence a cumulative over a number of generations.[15, 17, 21, 22, 23] Thus, two seeming widely separated fields, namely gerontology and cancer research, have no become interrelated experimentally.

The purposes of the present study are: (1) to present further data on t effect of maternal age selection on cancer susceptibility and to indicate a trends which may be arising (the final analysis can be made only wh divergent selection has been resorted to exhaustively and an experime dealing with reverse selection should then be performed); (2) to study t relationship between cancer susceptibility and general biology; and (3) study and report phenomena due to factors other than maternal age selectio such as sex differences, on the occurrence of chemically induced tumors.

Materials and Methods

The sublines used in this study were the Prunt, 2-Prunt, 4-Prunt, a 5-Prunt. The origin of these sublines is schematically presented in FIGURE The pBr subline was segregated from the NHO stock in the F$_{12}$ generati or after 8 generations of methylcholanthrene injection. Genetically, t pBr line contains double recessive genes for pink eye, brown coat, and no agouti, and has the symbol ppbbaa. The pBr mice were continued many generations by brother-to-sister matings to assure a high degree homozygosity. In the F$_{17}$ generation (13 generations of methylchola threne injection), a brother-sister pair was separated as progenitors of t pBrunt (later shortened to Prunt) subline. The 4-Prunt subline was sep rated from the F$_{11}$ generation of the Prunt line. The 2-Prunt subline w separated from the pBr stock in the F$_{20}$ generation (16 generations of meth cholanthrene injection). In the F$_{4}$ generation of the 2-Prunts, a brothe sister mating was used to give rise to the 5-Prunt subline.

In the NHO and pBr stocks all animals were injected with the carcinoge whereas in the four new sublines, untreated (unt), the breeders were n injected with methylcholanthrene. As seen from FIGURE 1, the 4-Prunt su line eventually died out, while the other 3 descents are being continued at t present time. The Prunt subline is now in the F$_{20}$ generation, total inbree ing 37 generations ($F_{17} + F_{20} = F_{37}$); 2-Prunt in the F$_{30}$ generation, to inbreeding 50 generations ($F_{20} + F_{30} = F_{50}$); and the 5-Prunt in the generation, total inbreeding 38 generations ($F_{20} + F_{4} + F_{14} = F_{38}$).

The breeders for the Prunt and 2-Prunt sublines were selected from ea (first and second) litters. The 4-Prunt and 5-Prunt breeders were select from late (fifth, sixth, and later) litters. If there were any factor in the agi process affecting susceptibility to chemically induced tumors it would smitted to the breeders, which would subsequently transmit it to their rimental or injected offspring.

he method of selection toward early litters is similar to the present an trend of having fewer offspring; hence, the age of mothers at the time lren are born is fairly young on the average. The fact that early litters large and breed well, whereas late litters tend to be small and breed rly, accounts for the greater number of generations in the sublines selected ard early litters. Also, the time lapse before continuing the mating of

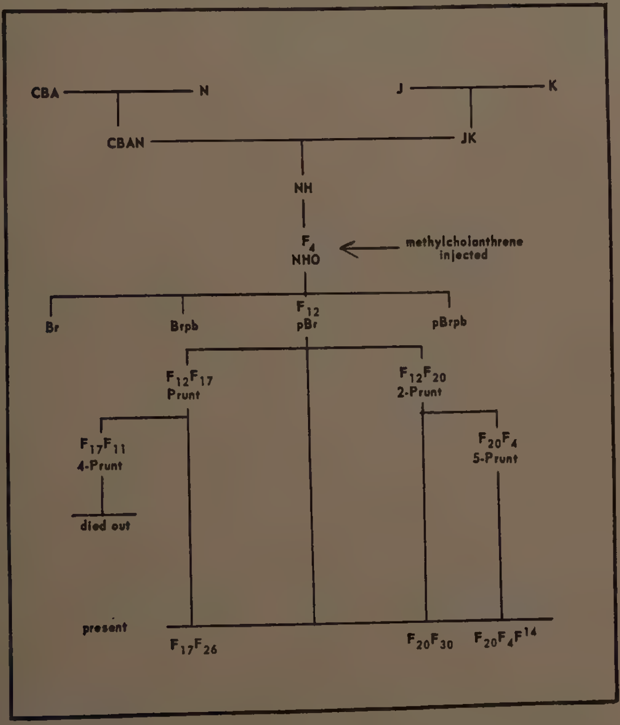

FIGURE 1. Origin of the sublines in the experiment.

ents of later descents is considerably greater than in the early litter cent—hence, fewer generations of inbreeding between 2-Prunts and runts.

n all the sublines the breeders, after being selected, were placed in separate es and allowed to breed as long as possible. The offspring were weaned approximately 30 days of age. At sixty + or − 3 days of age they were ected subcutaneously with 1 mg. of 20-methylcholanthrene dissolved in cc. sesame oil into the right flank. Injected mice were placed in boxes domly, with no attempt to separate sexes. Both sexes may or may not ve been in the same box. Any offspring born from injected animals were t saved. The injected animals were observed weekly for appearance of pable tumors. The latent period of the tumor was defined as the number

of days between injection and first gross appearance of a tumor. Surviv
time was the number of days between first appearance of the tumor and t
death of the animal. Animals were all autopsied after death and, when po
sible, specimens of tumors were preserved in Bouin's solution, embedded i
paraffin, and stained with hematoxylin and eosin. Animals were all giv
the same constant diet and kept in a fairly constant environment. All t
animals in the experiment were genetically similar, assuming a high degree
homozygosity following seventeen generations of inbreeding of the origin
pBr strain. Any difference should theoretically be due to selection, barri
a small amount of divergence due to spontaneous mutations, plus the fa
that complete homozygosity is not obtained (genetic segregation).

In the first ten generations of 2-Prunts the breeders were taken at rando
After ten generations the breeders were selected from first and second litte
only. The first ten generations were referred to as Series I and serves as o
series of controls for the experiment. Since some data for the 2-Prunt su
line have already been published,[23] data accumulated since publication pl
data not published before will be included in this paper.

It has long been recognized that painting a carcinogen on the skin of
animal produces a skin tumor, usually epidermoid carcinoma, whereas su
cutaneous injection produces neoplasia of connective tissue elements, usual
fibrosarcoma. Early in this experiment it became apparent that other h
tological types of tumors were occurring. Hence it became necessary
diagnose each tumor to rule out any difference attributable to differenc
in types of tumors.

Pathological specimens of the tumors were diagnosed and included if th
were malignant.

To date there have been diagnoses made on 3651 tumors occurring in 31
mice. This includes the period from approximately January 1949 to Ju
1956. The tumors were separated into the following categories:

(1) *Local tumors.* These, including tumors occurring at or near the si
of injection, were further subdivided into four histological types: (a) fibr
sarcoma, a diagnosis made whenever typical fibroblastic elements predom
nated and including a few tumors referred to as round cell sarcomas*
Strong and O'Neill;[23] (b) mammary gland carcinoma, including vario
histopathological types of mammary gland tumors such as medulla
intraductal, and scirrhous;[25] (c) squamous cell carcinoma, and (d) mix
tumors, including any tumors containing two or more neoplastic elemen
classified in the other three types of local tumors.

(2) *Lung tumors.* These included both primary and metastatic tumo

(3) *Liver tumors.* There were few liver tumors in this study and, wh
there were only two or three in the entire subline, they were included un
the heading "others."

(4) *Other tumors.* This class included rarer visceral growths such
stomach and kidney tumors.

Data collected for each type of tumor included latent period, survi

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* Since there were very few of these sarcomas in this study, and since they repres
anaplastic fibrosarcomas according to Boyd,[2] they were included as fibrosarcomas.

, sex of the animal, and litter to which it belonged. Tabulations were e for the following: (1) average latent period for each type of tumor; ercentage of incidence of each type of tumor in animals arranged in ps of fifties according to date of death (chronologically); (3) multiplicity umors; (4) latent period of tumor, survival time, and life span of the or-bearing animal for animals arranged in their respective litters; (5) entage of incidence of various tumors in animals arranged by litters; lung tumors, including percentage of incidence and latent period in the ous sublines; and (7) mammary gland tumors; latent period in virgins us breeding females. Results will be discussed for each subline and then comparative basis between the various sublines.

Results

he Prunt subline. In this subline there were 471 diagnosed tumors occur- in 377 female animals and 271 tumors in 258 males, making a total of diagnosed tumors in 635 animals. Tables 1 and 2 present the data on nt period and percentage of incidence of each type of tumor in females males respectively. Groups of 50 mice are arranged chronologically rding to date of death. The first class of 50 were the first 50 animals died, and so on down the table. Note that, for each group, local tumors prise 80 per cent to 90 per cent of total tumors. During the course of the eriment there has been no significant change in the percentage of incidence ny one type of tumor. The total number of tumors for each group is not ificantly different. Fibrosarcomas comprise, on the average, 55 per cent ll tumors in the females, but comprise an average of 83 per cent of all ors in males. It is interesting to note that local tumors other than osarcomas are rare in males, but plentiful in females. Hence, the female varied in the type of tissue susceptible to induced tumors. The com- ed percentage for all local tumors is practically the same for both sexes. igure 2 is a three-point moving curve depicting some trends in latent iods of various tumors during the course of the experiment. Note that latent period for each type of tumor has been increasing. This has been comitant with selection toward *early* litters. It would appear that ani- ls selected toward early litters become more resistant to induced tumors erms of latent period.

ince some animals bore more than one tumor, either histologically similar different, it was decided to make analyses on the basis of tumors rather n animals. This technique has been previously employed.[19]

able 3 represents the latent period of tumors, survival time, and life span tumor-bearing animals for animals arranged in litters. Figure 3 is a ee-point moving curve depicting the trends. Note that the latent period fibrosarcoma in females decreases with advancing litters, whereas in les it increases, and also that it is always, on an average, longer in males. e latent period for mammary gland tumors showed no trend in litter uence, but survival time decreased with advancing litters. Survival time animals bearing fibrosarcomas was practically a straight line. From ble 3 note that both the latent period and survival time for mammary

TABLE 1

PRUNT FEMALES: LATENT PERIOD IN DAYS AND PERCENTAGE OF INCIDENCE OF VARIOUS TUMORS IN ANIMALS ARRANGED IN GROUPS OF FIFTY CHRONOLOGICALLY ACCORDING TO DATE OF DEATH

Prunt females Animals in fifties		Local tumors				Lung	Liver	Other	To
		Fibro-sarcoma	Mam. gl. ca.	Squam. cell ca.	Mixed				
1–50	No. of tumors..	34	8	7	8	13	4	3	7
	% of total.....	44.2	10.4	9.1	10.4	16.9	5.2	3.9	100
	Lat. per., days.	145	196	107	157	216	173	326	—
51–100	No. of tumors..	33	6	8	10	6	1	—	6
	% of total.....	51.6	9.4	12.5	15.6	9.4	1.6	—	100
	Lat. per., days.	123	199	109	128	177	113	—	—
101–150	No. of tumors..	34	9	6	2	8	—	—	5
	% of total.....	57.6	15.3	10.2	3.4	13.5	—	—	10
	Lat. per., days.	124	136	113	113	129	—	—	—
151–200	No. of tumors..	38	11	1	7	3	1	1	6
	% of total.....	61.3	17.7	1.6	11.3	4.8	1.6	1.6	10
	Lat. per.. days.	158	137	141	194	162	115	402	—
201–250	No. of tumors..	34	8	4	5	9	1	—	6
	% of total.....	55.7	13.1	6.6	8.2	14.8	1.6	—	100
	Lat. per., days.	172	266	170	175	251	239	—	—
251–300	No. of tumors..	29	8	8	5	7	1	—	5
	% of total.....	50.0	13.8	13.8	8.6	12.1	1.7	—	100
	Lat. per., days.	150	199	181	111	209	306	—	—
301–377	No. of tumors..	58	9	8	2	13	—	—	9
	% of total.....	64.4	10.0	8.9	2.2	14.4	—	—	10
	Lat. per., days.	166	253	195	263	319	—	—	—
1–377	No. of tumors..	260	59	42	39	59	8	4	4
	% of total.....	55.2	12.5	8.9	8.3	12.5	1.7	0.8	10
	Av. lat. per., days........	150	195	122	156	225	183	345	—

gland tumors are longer than for fibrosarcomas in females. Note also th while the latent period for fibrosarcomas in females is shorter than in ma survival time is longer in females giving a life span (latent period plus survi time plus sixty days) not significantly different between the sexes. T would indicate that there are different mechanisms involved in latent peri and survival times for chemically induced tumors.

One must use caution in interpreting the data for litter seriation.

nber of animals in late litters is small; therefore, the size of the successive sses is not equal.

'ABLE 4 presents data on the percentage of incidence of various tumors in mals arranged by litters. The classification of tumor types was so chosen produce classes of significant size. There were no significant trends in centage of incidence of any type of tumor with advancing litter number. om the totals note that nearly 85 per cent of the tumors occurred locally.

TABLE 2

PRUNT MALES: LATENT PERIOD IN DAYS AND PERCENTAGE OF INCIDENCE OF VARIOUS TUMORS IN ANIMALS ARRANGED IN GROUPS OF FIFTY

Prunt males Animals in fifties		Local tumors				Lung	Other	Total
		Fibro-sar-coma	Mam. gl. ca.	Squam. cell ca.	Mixed			
–50	No. of tumors........	49	—	—	—	6	1	56
	% of total............	87.5	—	—	—	10.7	1.8	100.0
	Lat. per., days........	201	—	—	—	437	394	—
–100	No. of tumors........	44	—	1	—	6	3	54
	% of total............	81.5	—	1.9	—	11.1	5.6	100.0
	Lat. per., days........	161	—	588	—	347	181	—
1–150	No. of tumors........	41	1	3	2	1	—	48
	% of total............	85.4	2.1	6.3	4.2	2.1	—	100.0
	Lat. per., days........	172	142	175	132	795	—	—
1–200	No. of tumors........	44	—	—	—	8	1	53
	% of total............	83.0	—	—	—	15.1	1.9	100.0
	Lat. per., days........	204	—	—	—	469	567	—
1–258	No. of tumors........	49	—	1	—	10	—	60
	% of total............	81.7	—	1.7	—	16.7	—	100.0
	Lat. per., days........	187	—	81	—	502	—	—
1–258	No. of tumors........	227	1	5	2	31	5	271
	% of total............	83.8	0.4	1.8	0.7	11.4	1.8	100.0
	Av. lat. per., days....	185	142	238	132	460	301	—

f these locals in females, 65 per cent were fibrosarcomas. From TABLE 2 ote that over 95 per cent of local tumors in males were fibrosarcomas.

The 4-Prunt subline. This subline was selected toward a late litter descent. nce late litters are small in numbers, breeders were not plentiful and, hence, e number of offspring for experimental animals was restricted. This subne never bred well and eventually died out. There were 102 diagnosed mors in 82 females and 60 tumors in 59 males, making a total of 162 diagosed tumors in 141 animals.

Table 5 presents latent periods and percentage of incidences of various tumors. The animals of each sex were divided into two classes to see if there were any differences. There were no significant differences between the two groups for either sex. Note that fibrosarcomas comprised approximately 55 per cent of all tumors and 65 per cent of local tumors in females whereas, in males, fibrosarcomas comprised about 80 per cent of all tumors and more than 90 per cent of local tumors. The incidence of local tumors was the same for both sexes. Again the female shows greater variability in tissue susceptibility to induced tumors, namely, to bearing mammary gland tumors, squamous cell carcinomas, and mixed tumors. It is interesting to note the occurrence of three mammary gland tumors in the males. It is extremely rare to have such tumors in males induced by methylcholanthrene, or even under spontaneous conditions.

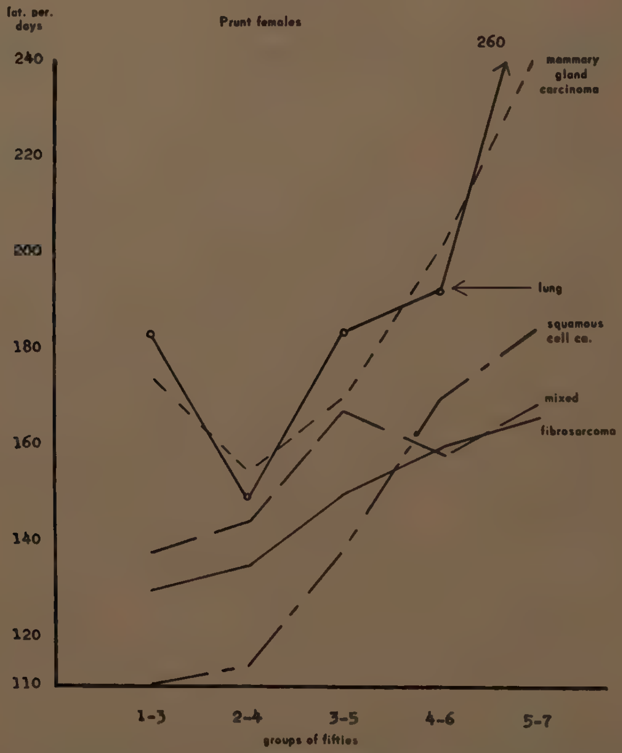

Figure 2. Prunt females: latent periods of various tumors in animals arranged by fifties. Three-point moving curve.

Table 6 presents data for animals arranged in litters. There were no significant trends with advancing litters. Since the classes were so small, it would be difficult to evaluate any trends that may have arisen. Note that the average latent period for fibrosarcomas in females was much shorter than that in males, while survival time was longer in females. Due to the longer latent period, the life span was longer in males.

The 5-Prunt subline. This subline, also selected toward late litters, was able to survive fairly well. There were 367 tumors diagnosed from 293 females and 193 tumors from 184 males, making a total of 560 tumors diagnosed from 477 animals. TABLES 7 and 8 summarize the data obtained. In the females the incidence of mammary gland tumors increased during the course of the experiment while the incidence of fibrosarcomas decreased.

TABLE 3

PRUNT: LATENT PERIOD, SURVIVAL TIME IN DAYS FOR TUMORS AND LIFE SPAN OF TUMOR-BEARING ANIMAL FOR ANIMALS ARRANGED BY LITTERS

Prunt Litter	Females								Males			
	Mammary gland carcinoma				Fibrosarcoma				Fibrosarcoma			
	No.	Lat. per., days	Surv. time, days	Life span, days	No.	Lat. per., days	Surv. time, days	Life span, days	No.	Lat. per., days	Surv. time, days	Life span, days
1	8	148	134	342	29	177	72	309	21	187	68	315
2	6	205	135	400	43	158	74	292	32	188	50	298
3	8	223	123	406	30	151	81	292	40	169	54	283
4	12	200	97	357	55	138	79	277	37	167	57	284
5	7	133	74	267	34	142	61	263	40	193	54	307
6	7	220	117	395	30	144	82	286	20	185	55	300
7	3	137	67	264	15	135	80	275	17	247	55	362
8	6	272	95	436	9	176	65	293	11	201	62	323
9	1	150	70	280	8	120	85	265	2	260	75	395
10	—	—	—	—	5	174	81	315	3	136	45	242
11	—	—	—	—	1	77	99	236	1	92	33	185
12	1	122	79	261	—	—	—	—	2	146	57	264
13	—	—	—	—	—	—	—	—	2	195	75	329
Average...		193	106	359		149	75	284		184	55	299
Total.....	59				259				228			

FIGURE 4 is a two-point moving curve depicting the trends in percentage of incidence of various tumors in females. The incidence of mammary gland tumors increased from 13 per cent to 42 per cent on the curve, while the incidence of fibrosarcomas decreased from 53 per cent to 36 per cent. This is an absolute inverse relationship rather than a relative one, since the number of tumors in each group of 50 animals is fairly constant. The incidence of other types of tumors did not alter significantly. As in the other sublines, local tumors comprised more than 80 per cent of all tumors in both sexes. Fibrosarcomas comprised about one half of all tumors in females, but more than 80 per cent in males. The difference was made up for by the other histological types of local tumors in the female. Again, the female showed variability as to the type of induced local tumor, whereas the male remained fairly constant. There were no significant trends in latent periods for the various tumors.

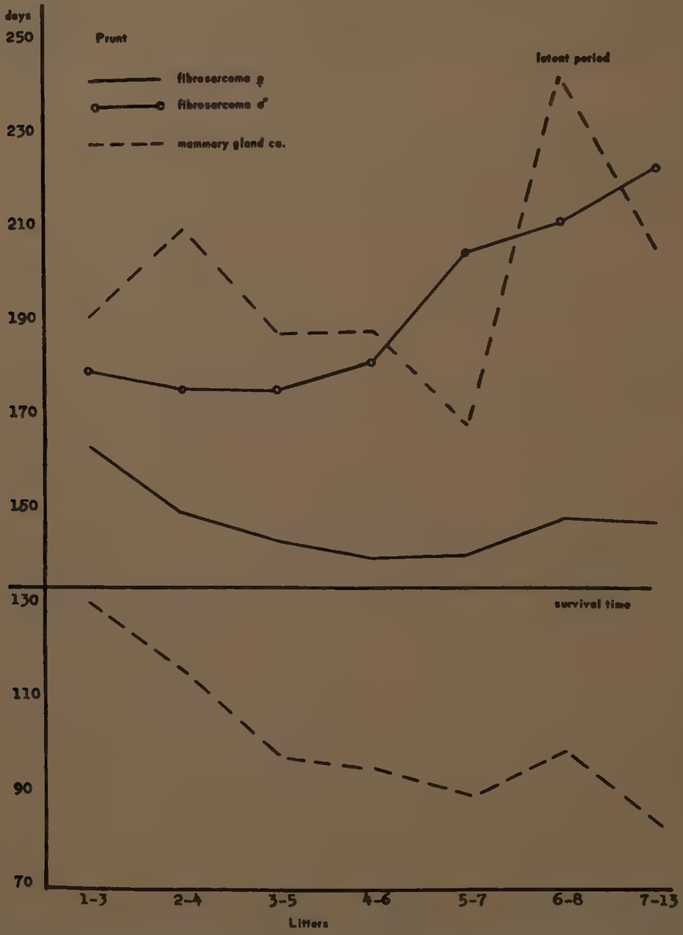

FIGURE 3. Prunt: latent period and survival time of tumors in animals arranged by litters. Three-point moving curve.

TABLE 9 presents data on tumors in animals arranged by litters. The trends are depicted in FIGURE 5, which is a three-point moving curve. The latent period for fibrosarcomas in both sexes, as well as for mammary gland carcinomas, increased with advancing litters. Survival time remained fairly constant throughout. Again the latent period for fibrosarcoma is longer in males, while survival time is longer in females. The male has a longer life span because of the longer latent period. The latent period and survival

TABLE 4
PRUNT: PERCENTAGE OF INCIDENCE OF TUMORS IN ANIMALS ARRANGED BY LITTERS

Prunt Litter	Females													Males					
	Local tumors								Total tumors					Total tumors					
	Fibro-sarcoma		Mam. gl. carcinoma		Other local		Total local		Local	Misc.		Total		Local		Misc.		Total	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1	29	67.4	8	18.6	6	14.0	43	100.0	82.7	9	17.3	52	100.0	21	87.5	3	12.5	24	100.0
2	43	75.4	6	10.5	8	14.0	57	100.0	90.5	6	9.5	63	100.0	32	74.4	11	25.6	43	100.0
3	30	62.5	8	16.7	10	20.8	48	100.0	84.2	9	15.8	57	100.0	43	95.6	2	4.4	45	100.0
4	55	65.5	12	14.3	17	20.2	84	100.0	84.0	16	16.0	100	100.0	39	86.7	6	13.3	45	100.0
5	34	56.7	7	11.7	19	31.6	60	100.0	89.6	7	10.4	67	100.0	41	89.1	5	10.9	46	100.0
6	30	69.8	7	16.3	6	14.0	43	100.0	74.1	15	25.9	58	100.0	20	80.0	5	20.0	25	100.0
7	15	62.5	3	12.5	6	25.0	24	100.0	88.9	3	11.1	27	100.0	17	89.5	2	10.5	19	100.0
8	9	56.3	6	37.5	1	6.3	16	100.0	80.0	4	20.0	20	100.0	11	84.6	2	15.4	13	100.0
9	8	57.1	1	7.1	5	35.7	14	100.0	93.3	1	6.7	15	100.0	2	66.7	1	33.3	3	100.0
10	5	83.3	—	—	1	16.7	6	100.0	85.7	1	14.3	7	100.0	3	100.0	—	—	3	100.0
11–13	1	25.0	1	25.0	2	50.0	4	100.0	80.0	1	20.0	5	100.0	5	100.0	—	—	5	100.0
Total.......	259	64.9	59	14.8	81	20.3	399	100.0	84.7	72	15.3	471	100.0	234	86.3	37	13.7	271	100.0

Table 5

4-Prunt: Latent Period and Percentage of Incidence of Tumors in Animals Separated into Two Classes

4-Prunt	Females						Males					
	Animals 1–41			Animals 42–82			Animals 1–29			Animals 30–59		
	No. tumors	Per cent total	Lat. per., days	No. tumors	Per cent total	Lat. per., days	No. tumors	Per cent total	Lat. per., days	No. tumors	Per cent total	Lat. per., days
Fibrosarcoma	29	56.9	114	28	54.9	133	24	85.7	176	25	78.1	252
Mammary gland carcinoma	2	3.9	107	4	7.8	98	2	7.1	179	1	3.1	274
Squamous cell carcinoma	6	11.8	118	5	9.8	90	—	—	—	1	3.1	434
Mixed	6	11.8	100	7	13.7	193	—	—	—	—	—	—
Lung	6	11.8	105	7	13.7	323	2	7.1	317	5	15.6	421
Other	2	4.0	102	—	—	—	—	—	—	—	—	—
Totals	51	100.0		51	100.0		28	100.0		32	100.0	

Table 6

4-Prunt: Latent Period, Survival Time for Tumors and Life Span of the Tumor-Bearing Animal for Animals Arranged by Litters

4-Prunt Fibrosarcomas	Females				Males			
Litter	No. tumors	Lat. per., days	Surv. time, days	Life span, days	No. tumors	Lat. per., days	Surv. time, days	Life span, days
1	5	121	89	271	9	194	33	308
2	13	117	64	242	13	207	47	314
3	15	137	83	280	9	256	53	370
4	11	125	56	241	7	255	58	373
5	6	130	79	269	4	183	56	299
6	4	111	65	237	4	174	48	282
7	1	81	90	231	—	—	—	—
8	2	106	91	256	2	206	67	333
9	—	—	—	—	1	155	40	255
Averages		124	73	257		215	52	327

time for mammary gland tumors were slightly longer than for fibrosarcomas in females, but not significantly so.

Table 10 presents data on the incidence of tumors in animals arranged by litters. There were no significant trends with advancing litter number. On the average, mammary gland tumors comprised nearly 30 per cent of local tumors. Fibrosarcomas comprised approximately 50 per cent of local tumors in females. From TABLE 8 note that fibrosarcomas comprise over 95 per cent of local tumors in males. Local tumors in this subline comprised more than 80 per cent of all tumors in both males and females.

The 2-Prunt subline. TABLE 11 presents data for the 2-Prunt females. Data on the first 800 animals in the table have been published.[23] It was decided to modify the table and use it with the data accumulated from June 1955 to June 1956 to show the trends depicted in FIGURES 6 and 7. Fibrosarcomas in the table include round cell sarcomas listed separately. This subline was composed of 2 series. The first 300 animals in the table comprise approximately the first series in which there was no selection of breeders based upon age. Selection toward early litters or early maternal age was

TABLE 7
5-PRUNT FEMALES: LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF TUMORS IN ANIMALS ARRANGED IN GROUPS OF FIFTY

5-Prunt females Animals in fifties		Local tumors				Lung	Other	Total
		Fibro-sarcoma	Mam. gl. ca.	Squam. cell ca.	Mixed			
1–50	No. of tumors	34	8	8	5	8	1	64
	% of total tum.	53.2	12.5	12.5	7.8	12.5	1.6	100.0
	Lat. per., days	127	152	116	149	127	142	—
51–100	No. of tumors	33	10	7	5	6	—	61
	% of total tum.	54.1	16.4	11.5	8.2	9.8	—	100.0
	Lat. per., days	138	137	169	158	162	—	—
101–150	No. of tumors	30	19	7	2	3	—	61
	% of total tum.	49.2	31.1	11.5	3.3	4.9	—	100.0
	Lat. per., days	122	140	97	90	196	—	—
151–200	No. of tumors	35	16	3	6	6	—	66
	% of total tum.	53.0	24.2	4.5	9.1	9.1	—	100.0
	Lat. per., days	158	168	128	129	270	—	—
201–250	No. of tumors	22	30	9	1	4	2	68
	% of total tum.	32.4	44.1	13.2	1.5	5.9	2.9	100.0
	Lat. per., days	133	139	119	181	328	342	—
251–293	No. of tumors	19	16	8	3	1	—	47
	% of total tum.	40.4	34.0	17.0	6.4	2.1	—	100.0
	Lat. per., days	151	157	106	122	62	—	—
1–293	No. of tumors	173	99	42	22	28	3	367
	% of total tum.	47.1	27.0	11.4	6.0	7.6	0.8	100.0
	Av. lat. per., days	138	148	121	138	200	275	—

exercised in Series II, which was composed of the other approximately 800 animals.

FIGURE 6 is a three-point moving curve depicting the latent periods of various tumors in Series II and is taken from TABLE 11, using animals from 301 to 1082. There has been an increase in latent period for most of the tumors during the course of the experiment similar to the Prunt subline.

FIGURE 7 depicts percentage of incidence only of mammary gland tumors and fibrosarcomas in the entire subline. Mammary gland tumors increased from 13 per cent to a maximum of 36 per cent, and then levelled off at 30 per cent. Fibrosarcoma showed an inverse relation by decreasing from 57 per cent to 40 per cent and then increasing to level off at 53 per cent. The incidence of the two types of tumors began converging until they became almost equal, and then diverged to approach equilibrium. This indicates a tendency on the part of the species to maintain equilibrium somehow in the face of a changing characteristic, in this instance a practically straight line increase in mammary gland carcinoma.

TABLE 8

5-PRUNT MALES: LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF TUMORS IN ANIMALS ARRANGED IN GROUPS OF FIFTY

5-Prunt males Animals in fifties		Local tumors			Lung tumors	Other tumors	Total
		Fibro-sarcoma	Mam. gl. ca.	Squam. cell ca.			
1–50	No. of tumors........	50	—	—	3	—	53
	% of total tum.......	94.3	—	—	5.7	—	100.0
	Lat. per., days.......	170	—	—	176	—	—
51–100	No. of tumors........	43	1	—	6	—	50
	% of total tum.......	86.0	2.0	—	12.0	—	100.0
	Lat. per., days.......	200	146	—	518	—	—
101–150	No. of tumors........	39	—	1	14	—	54
	% of total tum.......	72.2	—	1.9	25.9	—	100.0
	Lat. per., days.......	156	—	165	531	—	—
151–184	No. of tumors........	25	—	2	7	2	36
	% of total tum.......	69.4	—	5.6	19.4	5.6	100.0
	Lat. per., days.......	174	—	56	503	400	—
1–184	No. of tumors........	157	1	3	30	2	193
	% of total tum.......	81.3	0.5	1.6	15.5	1.0	100.0
	Av. lat. per., days....	175	146	92	486	400	—

The male animals are not reported again because there were no significant findings to report, other than those from the previous publication.[23] The number of data was increased, however, from 600 to about 800.

Comparative analysis of the four sublines. TABLE 12 presents data on incidence and latent periods of tumors in females of the 4 sublines as a whole. A total of 2302 tumors occurred in 1834 animals. The data for Series I of the 2-Prunts has been modified from a previous publication[23] and inserted here for purposes of comparison since it represents one control group (without selection). The most significant findings concern the incidence of mammary gland carcinomas. In the control group there were 12.6 per cent mammary gland tumors, while there were 31.0 per cent in Series II of the 2-Prunts and 27.0 per cent in the 5-Prunts. These differences were analyzed statistically. The test of significance used was the critical ratio that is equal to the difference between 2 quantities divided by the standard error of the difference. A critical ratio of 2 or more is statistically significant at the 0.05 level. The critical ratio for the difference between Series I and Series II was 3.4. The critical ratio for the difference between Series I and the 5-Prunts was 2.2. Both these differences were significant at the 0.05 level.

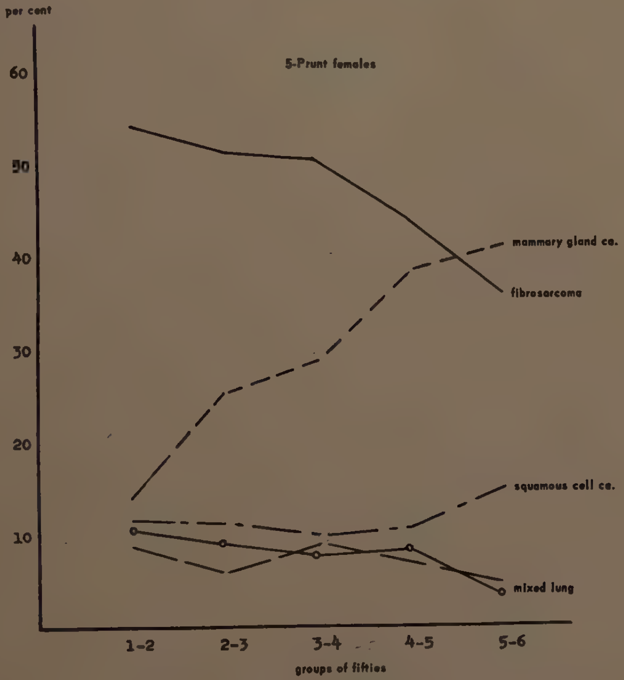

FIGURE 4. 5-Prunt females: percentage of incidence of various tumors. Two-point moving curve.

We may not attribute the increase in mammary gland tumors to maternal age selection since the 2 groups with the high incidence was selected in different directions. However, since the 5-Prunts were derived from the 2-Prunts, there may be some factor which arose before the 5-Prunts were separated off to account for the difference in tissue susceptibility to induced tumors.

The high incidence of mammary gland tumors was compensated for in the two sublines concerned by a decreased incidence of all other types of tumors. The decrease was distributed so that, even though there was a lower incidence of fibrosarcomas in Series II and in the 5-Prunts, statistically it was not significantly different from the controls (Series I of the 2-Prunts).

TABLE 9

5-PRUNT: LATENT PERIOD, SURVIVAL TIME IN DAYS FOR TUMORS AND LIFE SPAN OF TUMOR-BEARING ANIMAL FOR ANIMALS ARRANGED BY LITTERS

5-Prunt Litter	Females								Males			
	Mammary gland carcinoma				Fibrosarcoma				Fibrosarcoma			
	No.	Lat. per., days	Surv. time, days	Life span, days	No.	Lat. per., days	Surv. time, days	Life span, days	No.	Lat. per., days	Surv. time, days	Life span, days
1	31	146	80	286	34	138	80	278	45	170	55	285
2	25	142	76	278	48	134	75	269	31	177	50	287
3	20	131	102	293	29	133	81	274	33	171	58	289
4	7	148	54	262	27	140	89	289	21	188	60	308
5	7	181	94	335	20	160	85	305	19	184	52	296
6	4	176	74	310	5	131	62	253	3	199	56	315
7	1	166	159	385	3	143	60	263	3	209	68	337
8	3	134	57	251	5	128	58	246	—	—	—	—
9	1	207	100	367	3	167	115	342	2	134	43	237
Av........		146	83	289		134	77	271		177	55	292
Total.....	99				174				157			

There were no significant differences in the latent periods for any one type of tumor between the sublines. The latent period for fibrosarcoma in late litter descents (4-Prunt and 5-Prunt) was less than for early litter descents, but the difference was not statistically significant.

The latent period for mammary gland tumors was longer than for fibrosarcomas in general, and the latent period for lung tumors was longest. Lung tumors will be analyzed and discussed later. It is interesting to note on the bottom line of TABLE 12 that the averge number of tumors per animal for each subline was amazingly similar.

In TABLE 13 the data for tumors occurring in male animals are presented for the 4 sublines. There was a total of 1349 tumors in 1301 animals. Data on approximately 600 animals of the 2-Prunt subline have been reported previously.[23] The data here represent modifications and the addition of approximately 200 more animals. There were no significant differences noted between the sublines for any characteristic in male mice.

Fibrosarcomas comprised over 80 per cent of all tumors and practically 100 per cent of local tumors. To date, 12 mammary gland carcinomas have occurred in 9 male mice. In addition, a spontaneous mammary gland tumor occurred in a 2-Prunt male breeder. This does not appear in the data, since breeders are not included in this report. The first 10 mammary gland tumors in males have been reported.[9] The lung tumors comprised the only other significantly large group. Other types of tumors were quite rare, comprising 5 per cent of all tumors exclusive of fibrosarcomas and lung tumors. The average number of tumors per animal was again quite constant.

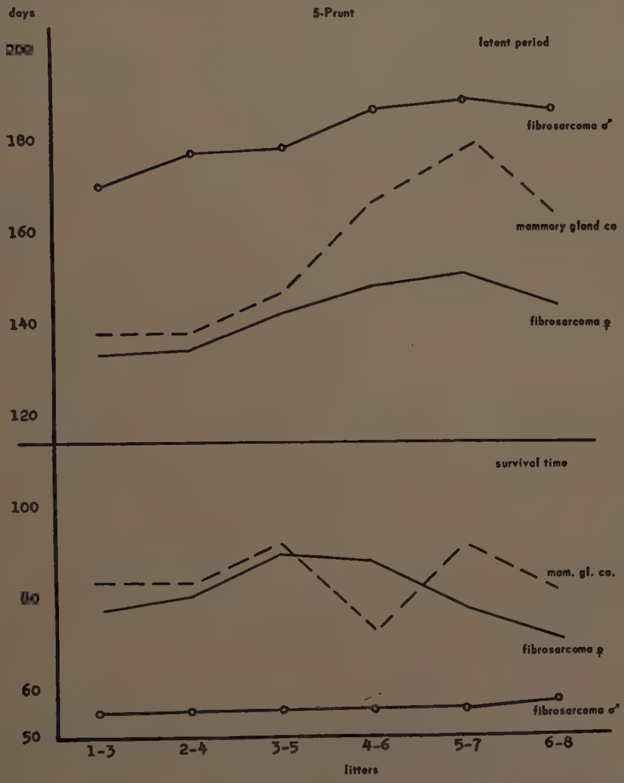

FIGURE 5. 5-Prunt: latent period and survival time of tumors in animals arranged by litters. Three-point moving curve.

Comparing TABLES 12 and 13, there were several sex differences noted. Females produce a higher incidence of local tumors other than fibrosarcomas. However, as stated before, the incidence for all local tumors combined is the same for both sexes. The latent periods for both fibrosarcomas and lung tumors were much longer in the male than in the female. This would indicate that females are more susceptible to induced tumors than males.

TABLE 10
5-PRUNT: PERCENTAGE OF INCIDENCE OF TUMORS IN ANIMALS ARRANGED BY LITTERS

5-Prunt Litter	Females													Males					
	Local tumors								Total tumors					Total tumors					
	Fibro-sarcoma		Mam. gl. ca.		Other local		Total local		Local	Misc.		Total		Local		Misc.		Total	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
1	34	43.0	31	39.2	14	17.7	79	100.0	92.9	6	7.1	85	100.0	45	80.4	11	19.6	56	100.0
2	48	54.5	25	28.4	15	17.0	88	100.0	93.6	6	6.4	94	100.0	31	86.1	5	13.9	36	100.0
3	29	44.6	20	30.8	16	24.6	65	100.0	87.8	9	12.2	74	100.0	36	87.8	5	12.2	41	100.0
4	27	61.4	7	15.9	10	22.7	44	100.0	93.6	3	6.4	47	100.0	22	78.6	6	21.4	28	100.0
5	20	64.5	7	22.6	4	12.9	31	100.0	88.6	4	11.4	35	100.0	19	82.6	4	17.4	23	100.0
6	5	50.0	4	40.0	1	10.0	10	100.0	83.3	2	16.7	12	100.0	3	100.0	—	—	3	100.0
7	3	75.0	1	25.0	—	—	4	100.0	80.0	1	20.0	5	100.0	3	100.0	—	—	3	100.0
8	5	45.4	3	27.3	3	27.3	11	100.0	91.7	1	8.3	12	100.0	—	—	—	—	—	—
9–10	3	75.0	1	25.0	—	—	4	100.0	100.0	—	—	4	100.0	2	66.7	1	33.3	3	100.0
Total.......	174	51.8	99	29.5	63	18.8	336	100.0	91.3	32	8.7	368	100.0	161	83.4	32	16.6	193	100.0

There was a difference in the number of tumors per animal. The critical ratio for the difference between the 2 sexes was 16, making it very significant statistically. This would also indicate a higher susceptibility to induced tumors in the female.

TABLE 11

2-PRUNT FEMALES: LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF TUMORS IN ANIMALS ARRANGED IN GROUPS OF ONE HUNDRED

2-Prunt females Animals in hundreds and No. of tumors		Local tumors				Lung tumors	Liver tumors	Other tumors
		Fibro-sarcoma	Mam. gl. ca.	Squam. cell ca.	Mixed tumors			
1–100 123 tumors	Percentage of incidence Lat. per., days	56.1 141	9.8 149	13.8 114	7.3 140	4.9 121	2.4 216	5.7 148
101–200 138 tumors	Percentage of incidence Lat. per., days	60.0 131	8.6 148	9.3 94	7.9 153	7.9 143	2.9 112	3.5 104
201–300 121 tumors	Percentage of incidence Lat. per., days	54.5 170	20.7 118	4.1 139	8.3 133	9.1 223	1.7 152	1.7 154
301–400 123 tumors	Percentage of incidence Lat. per., days	57.7 123	25.2 118	4.9 78	4.9 100	6.5 160	0.8 170	— —
401–500 127 tumors	Percentage of incidence Lat. per., days	52.0 112	22.8 131	6.3 99	3.9 130	13.4 172	1.6 79	— —
501–600 133 tumors	Percentage of incidence Lat. per., days	38.4 139	34.6 119	10.5 149	6.8 111	9.8 205	— —	— —
601–700 135 tumors	Percentage of incidence Lat. per., days	40.7 135	34.1 137	5.2 178	3.7 140	12.6 211	0.7 136	3.0 223
701–800 140 tumors	Percentage of incidence Lat. per., days	41.4 178	37.9 176	5.0 97	5.0 111	10.0 357	0.7 107	— —
801–900 121 tumors	Percentage of incidence Lat. per., days	47.9 142	30.6 212	7.4 174	— —	11.6 351	— —	2.5 250
901–1000 107 tumors	Percentage of incidence Lat. per., days	73.8 161	19.6 200	1.9 125	0.9 151	3.7 309	— —	— —
1001–1082 94 tumors	Percentage of incidence Lat. per., days	41.5 171	41.5 223	7.4 158	1.1 245	8.5 314	— —	— —
1–1082 Totals 1362 tumors	Percentage of incidence Av. lat. per., days	51.0 144	25.8 162	7.0 158	4.7 131	9.0 235	2.5 155	

In TABLE 14 the multiplicity of fibrosarcomas and mammary gland tumors is analyzed. A multiple tumor is defined as one occurring in an animal having more than one tumor. Thus if an animal had more than one tumor, each tumor was classified as multiple. FIGURE 8 depicts the data graphically.

There were no differences between the sublines for any characteristic. The ratio of multiple fibrosarcomas to single ones was approximately 1:3 for females. The ratio in males was app. 1:15. The critical ratio for the difference of the percentage of multiple fibrosarcomas between the 2 sexes was 12. More than one half of all mammary gland tumors were multiple. The difference between the percentage of multiple fibrosarcoma in females and that of mammary gland tumors had a critical ratio of 5.

The difference between the number of tumors per animal for males versus females noted before was not due solely to the greater number of mammary gland tumors in the female, but also to a higher multiplicity of fibrosarcomas in females. The data from TABLE 14 would indicate that females are more susceptible to induced tumors than males, and that mammary gland tissue is more susceptible than connective tissue to chemically induced malignant tumor formation in this inbred strain.

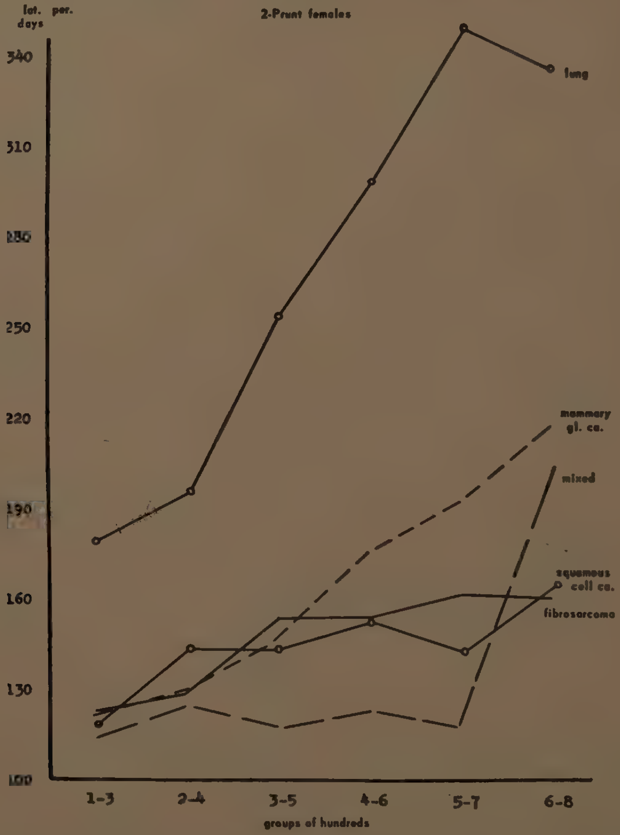

FIGURE 6. 2-Prunt females Series II: latent period of tumors in animals arranged by hundreds. Three-point moving curve.

Lung tumors were analyzed separately. In TABLE 15 lung tumors were classified as primary or metastatic for both sexes in all sublines. In practically every instance primary tumors were mature bronchogenic adenocarcinomas. The metastatic tumors were so classified because in each instance a histologically similar tumor was found on the surface of the animal. Andervont[1] has reported induced lung tumors when histologically similar tumors appeared at the site of administration of carcinogen on the surface of the animal. The metastatic tumors were assigned the latent period of the local tumor in the animal.

The latent period for primary tumors of the lung was arbitrarily calculated as the number of days from injection until the date of death, minus 30 days. Hence, not the actual latent period, but the fact of being a defined quantity, serves as a basis for comparison. FIGURE 9 is a histogram depicting the incidence of primary versus metastatic lung tumors.

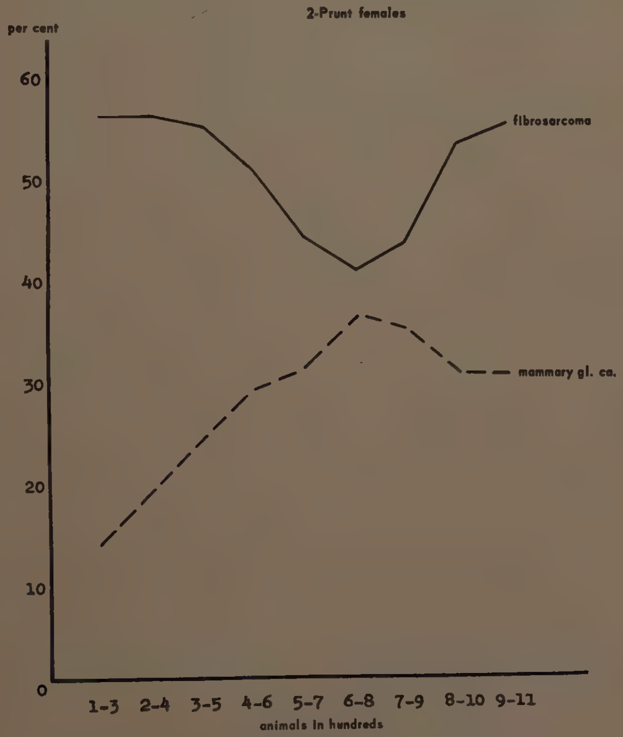

FIGURE 7. 2-Prunt females: percentage of incidence of tumors in animals arranged by hundreds. Three-point moving curve.

There were no significant differences between sublines, but there were some significant sex differences. An average of 33 per cent of all lung tumors in females were primary, whereas males had an incidence of 85 per cent primary. The difference was statistically significant. The latent period for primary lung tumors was shorter in the females than in the males. Females, being more susceptible to local induced tumors, do not live long enough to develop primary lung tumors. However, those females that do develop primary lung tumors have a shorter latent period than males because the females, over all, are more susceptible to induced tumors. This higher incidence of primary lung tumors in males is interesting, since it is analogous to the human situation.

TABLE 12

FEMALES OF ALL SUBLINES: LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF TUMORS

Females	Prunt			2-Prunt						4-Prunt			5-Prunt			Totals	
				Series I			Series II										
	No. tu- mors	Per cent of total	Lat. per., days	No. tu- mors	Per cent of total	Lat. per., days	No. tu- mors	Per cent of total	Lat. per., days	No. tu- mors	Per cent of total	Lat. per., days	No. tu- mors	Per cent of total	Lat. per., days	No. tu- mors	Per cent of total
Fibrosarcoma..........	260	55.2	150	220	56.7	149	475	48.8	143	57	55.9	124	173	47.1	138	1185	51.5
Mam. gl. ca..........	59	12.5	195	49	12.6	162	302	31.0	162	6	5.9	101	99	27.0	148	515	22.4
Squam. cell ca.........	42	8.9	122	35	9.0	120	60	6.2	131	11	10.8	106	42	11.4	121	190	8.3
Mixed tumors.........	39	8.3	156	30	7.7	142	34	3.5	122	13	12.7	150	22	6.0	138	138	6.0
Lung tumors..........	59	12.5	225	32	8.3	216	91	9.3	241	13	12.7	223	28	7.6	200	223	9.7
Liver tumors..........	8	1.7	183	8	2.1	161	—	—	—	1	1.0	54	—	—	—	17	0.7
Others..............	4	0.8	345	14	3.6	133	12	1.2	178	1	1.0	151	3	0.8	275	34	1.5
Totals...........	471	100.0	—	388	100.0	—	974	100.0	—	102	100.0	—	367	100.0	—	2302	100.0
No. of animals........	377			308			774			82			293			1834	
Tumors/animal........	1.25			1.26			1.26			1.24			1.25			1.255	

TABLE 13

MALES OF ALL SUBLINES: LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF TUMORS

Male mice	Prunt			2-Prunt			4-Prunt			5-Prunt			Totals	
	No. tumors	Per cent of total	Lat. per., days	No. tumors	Per cent of total	Lat. per., days	No. tumors	Per cent of total	Lat. per., days	No. tumors	Per cent of total	Lat. per., days	No. tumors	Per cent of total
Fibrosarcoma.........	227	83.8	185	679	82.3	179	49	81.7	215	157	81.3	175	1112	82.4
Mammary gland ca....	1	0.4	142	7	0.8	221	3	5.0	211	1	0.5	146	12	0.9
Squamous cell ca......	5	1.8	238	3	0.4	137	1	1.7	434	3	1.6	92	12	0.9
Mixed tumors.........	2	0.7	132	5	0.6	184	—	—	—	—	—	—	7	0.5
Lung tumors..........	31	11.4	460	109	13.2	464	7	11.7	391	30	15.5	486	177	13.1
Others..............	5	1.8	301	22	2.7	306	—	—	—	2	1.0	400	29	2.1
Totals...........	271	100.0	—	825	100.0	—	60	100.0	—	193	100.0	—	1349	100.0
No. of animals........	258			800			59			184			1301	
Tumors/animal.......	1.05			1.03			1.02			1.05			1.037	

The metastatic tumors were classified histologically because it was suspected that the difference for percentage of incidence between males and females was due to the high incidence of mammary gland tumors in females that frequently metastasize to the lung. The data are contained in TABLE 16. Fibrosarcoma was the tumor type that most frequently metastasized. A comparison of TABLES 16 and 12 makes it clear that various types of local tumors metastasized to the lung approximately as frequently as they occurred on the surface of the animal. Hence, the frequency of metastatic lung tumors in females must be explained by some mechanism other than frequency of mammary gland carcinoma.

TABLE 14
MULTIPLICITY OF TUMORS IN THE SUBLINES AS A WHOLE

Sublines	Females						Males		
	Mammary gland carcinoma			Fibrosarcoma			Fibrosarcoma		
	Mult.	Single	Total	Mult.	Single	Total	Mult.	Single	Total
Prunt									
No. of tumors.....	28	31	59	54	205	259	22	205	227
Per cent of total...	47.5	52.5	100.0	20.8	79.2	100.0	9.7	90.3	100.0
2-Prunt Series I									
No. of tumors.....	22	27	49	57	163	220	15	269	284
Per cent of total...	44.9	55.1	100.0	25.9	74.1	100.0	5.3	94.7	100.0
2-Prunt Series II									
No. of tumors.....	181	121	302	113	361	474	22	373	395
Per cent of total...	60.0	40.0	100.0	23.8	76.2	100.0	5.6	94.4	100.0
4-Prunt									
No. of tumors.....	4	2	6	11	46	57	3	46	49
Per cent of total...	66.7	33.3	100.0	19.3	80.7	100.0	6.1	93.9	100.0
5-Prunt									
No. of tumors.....	51	48	99	46	127	173	11	145	156
Per cent of total...	51.5	48.5	100.0	26.6	73.4	100.0	7.1	92.9	100.0
Totals									
No. of tumors.....	286	229	515	281	902	1183	73	1038	1111
Per cent of total...	55.5	44.5	100.0	23.8	76.2	100.0	6.6	93.4	100.0

During the course of analysis of data it was noted that some mammary gland tumors had very long latent periods. Upon further investigation, data presented in TABLE 17 were obtained. When the animals were injected at sixty days of age with methylcholanthrene they were randomly placed in boxes, with no attempt to keep sexes separate. Thus, by chance some females were put in with males, and in a few instances there were some boxes containing only females. The injected females were allowed to breed if males were in the box, but the offspring were not saved (forced breeding). Thus, some females were going through the reproductive cycle, while some remained virgin. Mammary gland tumors were classified as to whether they occurred in virgin or breeding mice, and the average latent period was calculated. Data are presented in TABLE 17 and depicted by a histogram in

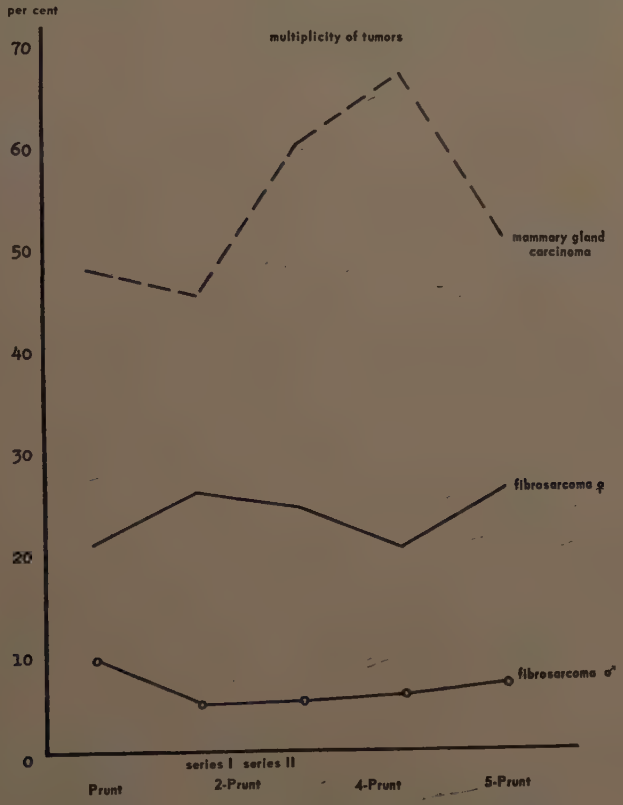

FIGURE 8. Multiplicity of tumors in the sublines.

FIGURE 10. TABLE 17 shows that virgin mice were fewer than breeding mice. These breeding mice must not be confused with breeders which were not injected and were used to produce offspring to be injected with carcinogen. The latent periods are recorded for one tumor in each mouse, that is, if a mouse had more than one mammary gland tumor, only one was included. This was done because it was desired to analyze differences between animals rather than tumors.

In Series I of the 2-Prunts the latent period for mammary gland tumors appearing in virgin mice was less than for those occurring in breeding mice.

The difference, 41 days, was not quite significant at the 0.05 level, the critical ratio being 1.8. In Series II and in the Prunt subline, the latent period was much longer in virgins than in breeding females. The differences in latent periods, 204 days and 67 days, respectively, were statistically significant. Thus, in animals selected toward early litter descents, virgin mice developing mammary gland tumors had a significantly longer latent period than did breeding females. This is more significant in view of the fact that in the control group virgin mice had a shorter latent period than breeding mice. The phenomenon of virgin females having longer latent periods than breeding females was reported many years ago,[8] and is confirmed in this study.

TABLE 15
LATENT PERIOD AND PERCENTAGE OF INCIDENCE OF LUNG TUMORS

Lung tumors		Females			Males		
Sublines		Primary	Metastatic	Total	Primary	Metastatic	Total
Prunt	No. of tumors....	16	37	53	25	5	30
	Per cent of total..	30.2	69.8	100.0	83.3	16.7	100.0
	Lat. per., days....	450	172	—	537	193	—
2-Prunt	No. of tumors....	46	77	123	92	15	107
	Per cent of total..	37.4	62.6	100.0	86.0	14.0	100.0
	Lat. per., days....	407	160	—	520	184	—
4-Prunt	No. of tumors....	3	9	12	7	0	7
	Per cent of total..	25.0	75.0	100.0	100.0	0.0	100.0
	Lat. per., days....	453	140	—	453	—	—
5-Prunt	No. of tumors....	6	21	27	23	5	28
	Per cent of total..	22.2	77.8	100.0	82.1	17.9	100.0
	Lat. per., days....	385	135	—	550	184	—
Totals	No. of tumors....	71	144	215	147	25	172
	Per cent of total..	33.0	67.0	100.0	85.5	14.5	100.0
	Lat. per., days....	417	159	—	525	186	—

Although not indicated in the table, virgin females had single mammary gland tumors in early litter descents, while breeding mice were prone to develop multiple mammary gland tumors. The above two observations would indicate that, in selection toward an early litter descent, virgin females become more resistant to induced mammary gland tumors than breeding females. Also, from the above one might think that these mammary gland tumors occurring in virgins were spontaneous rather than induced. However, according to one theory a carcinogen merely shortens the latent period for a tumor, since the animal already possessed the potential for developing it.[5]

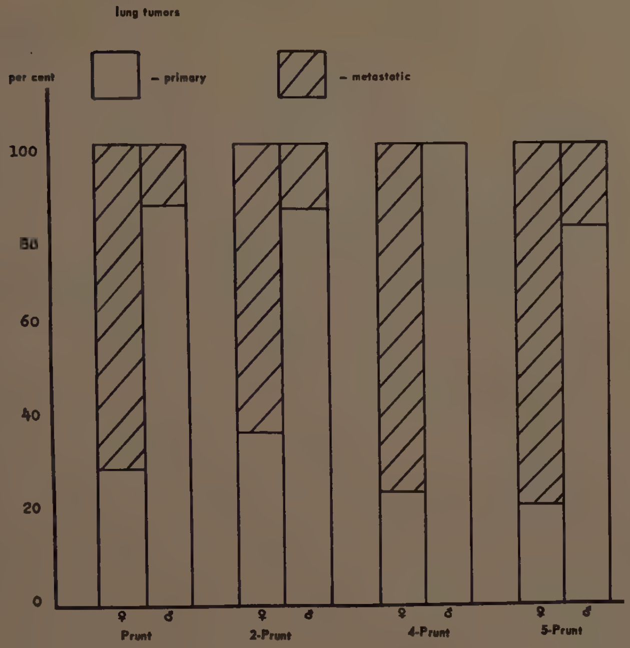

FIGURE 9. Percentage of primary and metastatic lung tumors.

TABLE 16
METASTATIC LUNG TUMORS IN FEMALE MICE

Metastatic lung tumors in females		Fibro-sarcoma	Mam. gl. ca.	Squam. cell ca.	Mixed	Total
Sublines						
Prunt	No. of tumors..............	21	6	9	1	37
	Per cent of total...........	56.8	16.2	24.3	2.7	100.0
2-Prunt	No. of tumors..............	37	26	9	5	77
	Per cent of total...........	48.1	33.8	11.7	6.5	100.0
4-Prunt	No. of tumors..............	7	1	—	1	9
	Per cent of total...........	77.8	11.1	—	11.1	100.0
5-Prunt	No. of tumors..............	12	7	—	2	21
	Per cent of total...........	57.1	33.3	—	9.5	100.0
Totals	No. of tumors..............	77	40	18	9	144
	Per cent of total...........	53.5	27.8	12.5	6.3	100.0

TABLE 17
ANALYSIS OF FEMALE MICE BEARING MAMMARY GLAND CARCINOMAS

Female mice c̄ mam. gl. ca.	2-Prunt						Prunt			5-Prunt		
Vir.: virgins Br.: breeding	Series I			Series II								
	Vir.	Br.	Total	Vir.	Br.	Total	Vir.	Br.	Total	Vir.	Br.	Total
No. of animals	14	31	45	20	202	222	18	37	55	6	77	83
Per cent of total	31	69	100	9	91	100	31	69	100	7	93	100
Lat. per., days	136	177	—	355	151	—	233	166	—	195	146	—
Difference, days	41			204			67			49		
Critical ratio	1.8			8.1			2.1			0.9		

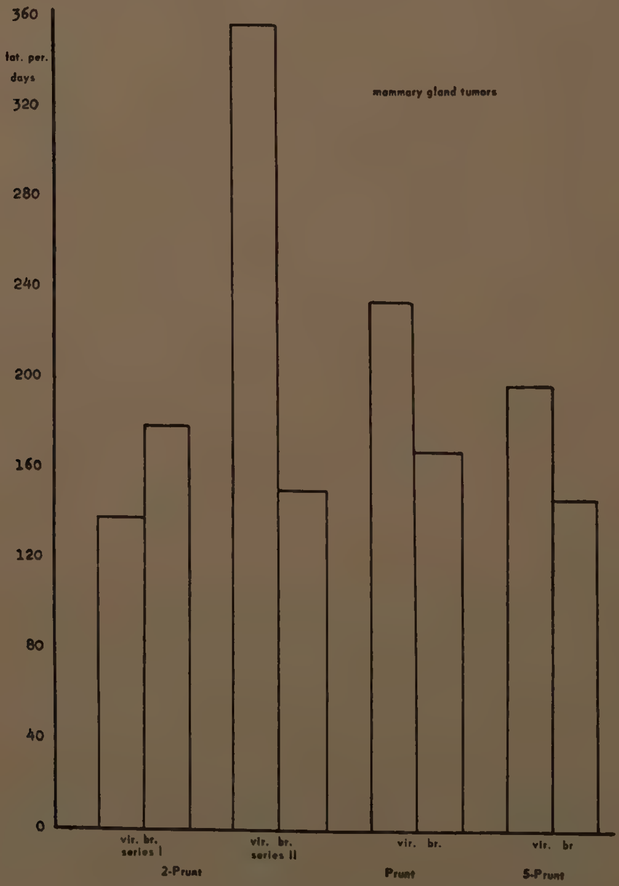

FIGURE 10. Latent period of mammary gland tumors.

In the 5-Prunt subline the latent period for tumors in virgins was longer than in breeders, but the difference was not statistically significant. There were only 6 virgins as compared to 77 breeding females, so that data were difficult to analyze statistically. Therefore, it is not known at present whether or not selection toward late litters has any greater effect on susceptibility to induced mammary gland tumors in virgins than in breeding females.

Since the experiment was not designed to study this phenomenon, the results of this last analysis must be interpreted with caution. However, the subject merits further investigation using many virgin mice to bring the classes to similar sizes so they can be analyzed statistically.

General Discussion

Results will be discussed under three headings corresponding to the three listed purposes of the experiment.

(1) *Effect of maternal age selection on cancer susceptibility.* From FIGURES 2 and 6 it has been shown that, as selection toward early litter descent continues, animals become more *resistant* to induced tumors as measured by an increase in latent period. With succeeding generations of inbreeding in animals of an early litter descent the age of the mother at the time offspring are born becomes continually younger.[24] Resistance to cancer susceptibility, however, increases; hence, the effect of selection is the reverse of the direction of selection.

From FIGURES 3 and 5 it can be seen also that increased resistance to cancer, as measured by longer latent periods or survival times, occurs in the litter numbers toward which selection is directed. The last two or three points on the curves are not too significant, since the numbers in these classes are small compared with those in the first part of the curves. Thus in Prunt females, (early litter descent) with advancing litters susceptibility to cancer increased, the early litters being more resistant. In the 5-Prunt subline (late litter descent) resistance to induced cancer increased with advancing litter number, later litters being more resistant.

It was noted in analyzing mammary gland tumors in the early litter descents (Prunt and Series II of 2-Prunts) that virgin animals were more resistant to this type of induced tumor than breeding animals. Why maternal age selection has any effect on this phenomenon is unknown. The observation is reported because it evoked interest and merits further investigation.

(2) *Relation of cancer susceptibility and other fields of biology.* It was noted from FIGURES 4 and 7 that the incidence of mammary gland tumors has increased in the 2-Prunt and 5-Prunt sublines. In the 2-Prunts the curve has leveled off, indicating restoration of equilibrium. The 2-Prunts contain 47 generations; the 5-Prunts, only 35 generations of inbreeding (21 generations of inbreeding is common). The point will probably be reached in the 5-Prunts when the incidence of mammary gland tumors will stop increasing and become more constant. This is one example of an attempt on the part of the species to maintain equilibrium.

As previously noted, selection toward shorter and shorter maternal age produced mice with longer and longer latent periods; the trend was in the reverse of the direction of selection. This, too, illustrates an attempt on the part of the species to maintain equilibrium in the face of (adverse) selection. A similar phenomenon was noted by Strong and Sanghvi;[19] when selection was used to try to obtain a strain resistant to cancer by selecting offspring from breeders with longer and longer latent periods, results were obtained opposite to those expected. As selection toward resistance continued, the mice became more susceptible as measured by shorter and shorter latent periods.

Strong has observed a similar phenomenon in an experiment using selection of litter size.[24] When mice were selected toward large litters the effect of selection was to produce a descent having smaller litters.

The above four observations represent accumulating data indicating that there are mechanisms at work that tend to keep the species more or less in equilibrium. It has been thought that by selection one could obtain a descent of animals all having a variant characteristic of an ancestor. This is true for simple Mendelian genetics. Accumulating evidence indicates that, using selection for characteristics not controlled by simple genetics, one obtains results to a certain degree and that then there is an attempt to restore a sense of balance offset by selection. In the past many investigators have been disappointed when experiments using selection failed to yield desired results. Their "failure" may have been due to this compensatory phenomenon on the part of the species to resist the effects of selection.

An interesting observation possibly related to the field of endocrinology was made concerning differences in susceptibility to mammary gland carcinoma between virgin and breeding females. Only in recent times have there been reports of mammary gland tumors induced by hydrocarbons.[4, 10] The rates of spontaneous mammary gland tumors in the ancestral stocks of the mice used in this experiment have not been high. A physiological basis for the higher incidence of mammary gland tumors in breeding females may be found in the fact that these animals are constantly undergoing the reproductive cycle and consequent cyclic changes in the mammary tissue. The effect of carcinogen plus physiological changes in the mammary tissue may combine to produce this increased incidence of tumor formation. In the human population a different mechanism must apply, since mortality from cancer of the breast is higher in unmarried (virgin) than married (breeding) females.

Another phenomenon observed in this study involves the concept of tissue susceptibility to chemically induced cancer. The production of the inbred strains in this study has led to the production of varying histological types of tumors at the site of injection. It is known that carcinogens applied topically or subcutaneously are absorbed somewhat, pass into the systemic circulation, and can be detected in urine and feces.[7] Thus, it is possible that different histological types of tumors are due to the spreading of the carcinogen. Since mammary gland tissue is widely distributed over the surface of the mouse it is obvious that carcinogen could come in contact with this ssue. It is also conceivable that epidermoid squamous cell carcinomas ould be elicited merely by infiltration of the carcinogen to the epidermis. f particular interest are the mixed tumors in which two or more definitely eoplastic elements occur. Evidently the carcinogen affects two tissue types ying in such close proximity that both become malignant at the same time.

From TABLE 12 it can be seen that the various types of surface tumors vary n latent periods. Squamous cell carcinoma seems consistently to have the hortest latent period, about 125 days. Mixed tumors and fibrosarcomas ad an intermediate latent period, about 140 to 150 days. Mammary gland umors had the longest latent period for local tumors. The latent period ould seem to indicate that squamous epithelium is most susceptible to hemically induced carcinogenesis and that mammary gland is most resistant. ercentage of incidence cannot be used as an index of tissue susceptibility ere because the carcinogen was injected directly into connective tissue nd/or, at times, mammary gland tissue.

The use of multiplicity of tumors as an index of susceptibility to induced umors makes it obvious that mammary gland tissue is most susceptible. s many as 4 and 5 mammary gland tumors were found in some animals. The question is whether these represented metastases from a single tumor, ndicated a multiple systemic response to carcinogen, or were due to subcutaneous infiltration of carcinogen, eliciting tumor formation at several iscrete points. At times, mammary gland tumors appeared far removed rom the site of injection. In some uninjected breeders several spontaneous mammary gland tumors appeared almost simultaneously at various places. The last two observations would indicate that there are many potential sites at which mammary gland tumors may occur, but this does not clarify the mode of action of the carcinogen.

Susceptibility of tissues to chemically induced tumors can therefore be viewed as regards: (1) latent period; (2) multiplicity of tumors. From data on mammary gland tumors it would seem that mammary tissue is comparatively resistant in terms of latent period, but susceptible in terms of multiplicity. Hence, different mechanisms influencing the two characteristics must be at work.

(3) *Sex differences.* There have been many reports indicating a sex difference for susceptibility to chemically induced tumors.[19, 20, 23] This study confirms observations that females are more susceptible to induced tumors than males. This is made evident by three criteria: (1) the latent period for fibrosarcomas is shorter in females; (2) females are more apt to have multiple tumors, that is, multiple fibrosarcomas as well as multiple mammary gland tumors; (3) different histological types of tumors develop in females. It is understandable that, due to estrogenic influences, mammary gland tumors may arise frequently in females but not in males. However, there was a tendency for squamous cell carcinomas and mixed tumors to occur more frequently in females. This makes it appear that there must be some factor, either physiological or linked genetically with sex, which renders various tissues more susceptible to induced tumors in females.

Survival times showed an interesting phenomenon. Females had longer survival times than males for fibrosarcomas. In both sexes the survival times were constant for all sublines and for all litters. The average survival time for males was 55 days and for females 75 days. The tumors were rather uniformly lethal or virulent in that once they formed, irrespective of latent period trends, animals died in a fairly constant span of time. Since females survived somewhat longer, it would seem that they are more resistant to the lethal effects of a malignant tumor. This may be another manifestation of greater resistance and adaptability of the female to stress situations.

Another sex difference was in the realm of lung tumors. Females appeared to be more susceptible to the occurrence of metastases. They were also more susceptible to primary tumors in terms of latent period. Thus, there must be some physiological or endocrinological factor influencing the process whereby surface tumors metastasize to lung. Since actual latent period and survival time cannot possibly be determined for each lung tumor, one must use caution in interpreting the defined latent period and using it as an index of susceptibility. If one assumes that primary lung tumors were spontaneous and not induced, it would appear that male animals are more susceptible to this type of tumor in terms of percentage of incidence. However, as stated previously, this may be due to the fact that females, being more susceptible to locally induced tumors, die younger on the average and do not live long enough to develop primary lung tumors.

Conclusions

(1) As selection toward an early litter descent continues over succeeding generations of inbreeding, animals become more resistant to chemically induced tumors as measured by an increase in latent period.

(2) In the case of females, at least, resistance to induced tumors is greatest in the litter numbers toward which the subline is selected. For example, early litters of the Prunt subline were more resistant to induced tumors than later litters of the same subline.

(3) Virgin animals in the early litter descents were more resistant to induced mammary gland tumors than breeding females, as measured by longer latent period.

(4) There are factors at work, genetic or otherwise, which tend to keep the species in a state of equilibrium such that selection in one direction elicits a compensatory response counter to the direction of selection.

(5) The susceptibility of various tissues to chemically induced tumor formation is influenced, to some degree, by the metabolism of the tissue and by hormonal factors, as evidenced by higher tissue susceptibility in female mice.

(6) Observations previously reported that female mice are more susceptible to chemically induced tumors than males were confirmed by this study.

(7) Different factors control latent period and survival time, since the latent period can be influenced by selection, but survival time is fairly constant for a given tumor type in a given sex.

(8) Female mice are more susceptible to pulmonary metastases from tumors occurring on the surface of the animal than males are. Primary lung tumors occur with greater frequency in male mice.

Summary

This experiment attempted to study the effect of certain variables, chiefly aternal age, on the susceptibility to methylcholanthrene-induced tumors n mice. The animals in the study consisted of a control group, two sublines elected toward early litters, and two sublines selected toward late litters. xperimental animals were injected subcutaneously with 20-methylchol-nthrene at 60 days of age.

Data were collected and analyzed from a total of 3651 pathologically iagnosed tumors occurring in 3135 animals. For comparison, data were resented for each subline and then for sublines as a whole. Results were iscussed under three headings: (1) effects of maternal age selection on cancer usceptibility; (2) relation of cancer susceptibility to general biology; and (3) ex differences. Conclusions were drawn to the effect that: (1) maternal age election does influence cancer susceptibility; (2) cancer susceptibility may e connected with a compensatory phenomenon that tends to keep the species n equilibrium; and (3) sex differences in cancer susceptibility that have been previously reported were confirmed.

Acknowledgment

For permission to use his experimental material in preparing this thesis I express my appreciation to Leonell C. Strong, a pioneer in cancer research whose study represents more than twenty years of planning, thinking, and experimentation.

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MAMMARY TUMORS IN AGENT-FREE MICE

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During the past several years sufficient data have been accumulated to ndicate that, although the presence of the mammary tumor agent may be a ery important factor, it is not essential for the appearance of all mammary umors in mice.[1-7] In 1950 we reported a substrain, then referred to as train C3Hb and now designated as C3Hf, which through foster nursing had been deprived of the mammary tumor agent. It was originated from a litter removed from the high-tumor strain C3H mother by cesarean section and oster-nursed upon a low-tumor strain C57BL female that did not contain the agent. Although it was anticipated that very few mammary tumors would occur in the females of this substrain, 38 per cent of the females of the first 5 generations that were intensively bred developed such tumors at an average age of 20 months.[6]

There was no evidence of the agent in cell-free filtrates of any of the 24 tumors of this substrain that were tested. Furthermore, the tumors occurred in females scattered throughout the pedigree chart rather than grouping in certain families segregating out as high-tumor lines. There was, however, a slight positive parent-offspring correlation, but it was recognized that this alone did not constitute proof of the presence of the agent, for slight variation in genetic factors or in extrachromosomal factors other than the agent could be expected to give such a correlation. It therefore appeared that this relatively high incidence of spontaneous mammary tumors had occurred in the absence of the agent because of the strong genetic influence and the intensive breeding.

Mammary tumors occurred in only 2 per cent of 100 females of this C3Hf substrain kept as virgins,[8] but when such virgin females were chronically irradiated the incidence of mammary tumors exclusive of sarcomas rose to 23 per cent.[9] Of a group of C3Hf males bearing pellets of cholesterol containing 10 per cent diethylstilbestrol, 20 per cent developed mammary tumors.[10]

This substrain C3Hf has been continued by brother × sister inbreeding, and complete data are now available on the tumors occurring in the breeding females from the F_6 through the F_{25} generation. In addition, three other substrains designated as C3Hff, C3Hf/2, and C3Hf/An also have been studied. Data on the occurrence of mammary tumors in these four substrains have been analyzed in an attempt to ascertain whether or not the agent is involved in the etiology of the tumors.

Substrain C3Hf

There have been a total of 911 females in generations F_6 through F_{25} of the breeding colony of this substrain. These mice, as well as those of the other 3 substrains, have been kept in an air-conditioned room at a temperature of approximately 78° F. Before 1949, at the time of the F_9 and F_{10} generations, the animals were housed in wooden cages. Since that time, they have been kept in plastic cages. Pine shavings were used for bedding until 1951, at the F_{13} to F_{15} generation; since then a mixture of one fourth cedar and three fourths pine shavings has been used. Routinely, the breeding cages were set up when the mice were weaned at 4 weeks of age, with 1 male and his litter mate sisters (usually 3 or 4) in each cage. When females were found to be pregnant they were isolated into separate cages and returned to the males after their litters were weaned. The breeding cages were maintained as long as the females bred; the females were then separated from the males and retired in cages with 8 females to each cage and were kept until they developed tumors or became moribund from other causes. At this time they were killed and examined for tumors and other lesions. Females found dead in the cages were likewise examined. The tumors were fixed in Fekete's modification of Tellyesniczky's fixative (70 per cent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) and were sectioned and stained with hematoxylin and eosin for histological examination.

TABLE 1

OCCURRENCE OF MAMMARY TUMORS IN BREEDING C3Hf FEMALES

Generation	Number of females	Avg. no. of litters	Incidence of mammary tumors (percentage)	Avg. tumor age (months)	Avg. age females died without tumor (months)
F_6	72	4.6	35	19.8	20.0
F_7	75	4.4	20	19.3	19.5
F_8	57	4.1	16	19.0	16.5
F_9	71	3.8	14	20.5	15.5
F_{10}	61	4.7	20	17.6	15.0
F_{11}	63	4.6	22	19.7	16.3
F_{12}	26	4.2	19	18.0	13.0
F_{13}	32	4.2	16	18.2	14.0
F_{14}	47	4.3	26	19.6	18.0
F_{15}	61	3.7	23	20.3	18.1
F_{16}	50	3.0	24	20.1	19.8
F_{17}	40	3.1	23	22.3	21.8
F_{18}	34	3.2	29	21.7	22.1
F_{19}	22	3.8	23	19.0	20.4
F_{20}	43	3.3	33	21.1	20.0
F_{21}	33	2.6	18	20.5	21.6
F_{22}	41	3.3	22	19.3	19.6
F_{23}	38	3.4	16	21.2	21.1
F_{24}	23	5.3	26	22.8	17.7
F_{25}	22	3.7	27	19.2	21.9
F_{6-25}	911	3.9	22	19.9	18.3

Mammary tumors occurring in the C3Hf females are listed in TABLE 1. The incidence of females with mammary tumors in the various generations varied from 14 per cent in F_9 to 35 per cent in F_6, with an incidence of 22 per ent for the total 911 females. The average age at which the tumors appeared varied from 18 months in F_{12} to 22.8 months in F_{24}, with an average of 19.9 months for all generations. The average age at which females without mammary tumors died varied from 13 months in generation F_{12} to 22.1 months in F_{18}, with an over-all average of 18.3 months. There was no evidence of a mammary tumor agent that was either dying out or being built up in successive generations, for neither the incidence of tumors nor the average tumor age showed any consistent increase or decline.

The incidence of mammary tumors in these F_6 to F_{25} generations was somewhat less than the incidence of 38 per cent observed in generations F_1 to F_5. However, the females of these later generations were not bred so intensively, having an average of 3.9 litters compared with 4.5 litters for the females of generations F_1 to F_5. As discussed more fully later, this could account in part at least for the percentage difference.

Substrain C3Hff

In order to gain additional evidence as to whether or not the mammary tumor agent was involved in the appearance of the tumors in the females of substrain C3Hf, a second substrain was started by foster nursing a litter of C3Hf young on a strain C57BL female without the agent. This fostered litter contained 2 males and 2 females, removed by cesarean section from their C3Hf mother, who was in generation F_9. It was assumed that if the agent were involved and the original foster nursing had reduced it in amount or potency so as to reduce the incidence of tumors from 97 per cent in the strain C3H breeding females to 38 per cent in the breeding C3Hf females, a second foster nursing should in turn significantly reduce the incidence of mammary tumors to some level below that in the substrain C3Hf. This second substrain was designated as C3Hff and was continued through 7 brother × sister generations.

Mammary tumors occurring in substrain C3Hff are listed in TABLE 2. Although the 2 original foster-nursed females died without mammary tumors, the incidence of females with mammary tumors in succeeding gen-

TABLE 2

OCCURRENCE OF MAMMARY TUMORS IN BREEDING C3Hff FEMALES

Generation	Number of females	Avg. no. of litters	Incidence of mammary tumors (percentage)	Avg. tumor age (months)	Avg. age females died without tumor (months)
F_1	2	5.5	0	—	17.5
F_2	24	4.6	21	16.0	15.2
F_3	18	5.3	28	19.8	17.7
F_4	19	4.3	37	18.1	18.3
F_5	28	4.6	39	18.5	19.4
F_6	18	3.3	22	22.0	20.1
F_7	9	2.3	22	23.0	20.0
F_{1-7}	118	4.3	29	18.9	18.2

erations ranged from 21 to 39 per cent, with an incidence of 29 per cent fo the 118 females in all 7 generations. The average age at which the tumor appeared varied from 16 months in generation F_2 to 23 months in generation F_7, with an over-all average of 18.9 months. The average age at which females of all 7 generations died without mammary tumors was 18.2 months This second foster nursing therefore did not result in a further reduction in tumor incidence, as might have been expected had the agent been present In fact, the incidence in the substrain C3Hff was somewhat higher than tha in the F_6 to F_{25} generations of the substrain C3Hf, but this was probably because the C3Hff females had more litters. The average number of litter for the C3Hff females was 4.3, compared with 3.9 for the C3Hf females The average tumor age for the C3Hff females was comparable with that o the C3Hf females.

Since the occurrence of mammary tumors in the C3Hff substrain was no significantly different from that in the C3Hf substrain, the C3Hff was no continued beyond the F_7 generation.

Substrain C3Hf/2

In order to determine whether or not the incidence of tumors in substrain C3Hf was specific for this substrain or was what could be expected in other substrains originated in the same manner, another substrain, designated as C3Hf/2, was started. This substrain was begun in 1954, at which time the C3Hf substrain was in generations F_{23} and F_{24}. As with the starting of C3Hf, a litter consisting of 1 female and her male littermates was removed from the C3H mother by cesarean section and foster nursed on a C57BL female. Later this foster-nursed female was mated with 1 of her male littermates, and the substrain was continued by brother × sister inbreeding for 5 generations.

Mammary tumors occurring in the substrain C3Hf/2 females are listed in TABLE 3. A mammary tumor occurred in this original female when she was 15 months of age, and the incidence for all females of the 5 generations was 51 per cent, with an average tumor age of 20.2 months. Females without mammary tumors died at an average age of 22.8 months. This incidence

TABLE 3

OCCURRENCE OF MAMMARY TUMORS IN BREEDING C3Hf/2 FEMALES

Generation	Number of females	Avg. no. of litters	Incidence of mammary tumors (percentage)	Avg. tumor age (months)	Avg. age females died without tumor (months)
F_1	1	6	100	15.0	—
F_2	6	6.2	17	18.0	24.4
F_3	27	5.6	59	20.8	22.7
F_4	23	6.1	52	20.3	21.5
F_5	15	5.9	47	19.6	23.8
F_{1-5}	72	5.8	51	20.2	22.8

is higher than that in the C3Hf or the C3Hff substrains, but this is probably due to the fact that the C3Hf/2 females lived a little longer and were bred more intensively than the C3Hf or C3Hff females. The average number of litters for the C3Hf/2 females was 5.8, compared with 4.3 for the C3Hff females and 3.9 for the C3Hf females of generations F_6 to F_{25}. The incidence of C3Hf/2 females with mammary tumors was also higher than the 38 per cent incidence in the C3Hf females of generations F_1 to F_5, but these females had an average of 4.5 litters, which also was less than that for the C3Hf/2 females.

The C3Hf/2 substrain was not continued beyond the F_5 generation.

Substrain C3Hf/An

During this time H. B. Andervont was maintaining a line of strain C3H without the mammary tumor agent that had been started in a manner similar to the origin of substrain C3Hf, but in which he had observed only 3 per cent with mammary tumors. His line was related to ours in that our strain C3H mice had been derived from his strain C3H in 1941. In 1942 he started his agent-free line from his C3H colony, and in 1945 we started substrain C3Hf from a C3H female in the seventh generation of inbreeding subsequent to the origin of our strain C3H from his colony. In 1953, in order to make a better comparison between Andervont's agent-free line and our substrain C3Hf, a litter containing 5 females and 2 males was obtained from his line in the F_{26} generation of inbreeding, and from this litter a line designated as substrain C3Hf/An was started and continued in our animal room with the same care and breeding system as used with our other agent-free substrains. We continued the substrain C3Hf/An from the F_{26} generation through the F_{31} generation.

Mammary tumors occurring in the C3Hf/An females are listed in TABLE 4. A mammary tumor occurred in one of the original 5 females at 12 months of age, and the incidence in the 96 females of generations F_{26} through F_{31} was 27 per cent, with an average tumor age of 19.9 months. The females without mammary tumors died at an average age of 20.3 months.

TABLE 4
OCCURRENCE OF MAMMARY TUMORS IN BREEDING C3Hf/An FEMALES

Generation	Number of females	Avg. no. of litters	Incidence of mammary tumors (percentage)	Avg. tumor age (months)	Avg. age females died without tumor (months)
F_{26}	5	4.2	20	12.0	25.5
F_{27}	7	4.0	14	20.0	17.0
F_{28}	22	5.6	32	17.6	17.7
F_{29}	35	6.1	26	22.1	20.5
F_{30}	23	6.0	30	21.0	21.9
F_{31}	4	5.5	25	17.0	23.7
F_{26-31}	96	5.7	27	19.9	20.3

Thus a relatively high incidence of mammary tumors occurred also in this agent-free substrain. The incidence was slightly higher than that in the substrain C3Hf females of generations F_6 to F_{25}, but possibly not as much higher as might have been expected, since the C3Hf/An females had on the average 5.7 litters compared with only 3.9 for the C3Hf females. The C3Hf/2 females that compared closely with the C3Hf/An females in respect to number of litters had an incidence of mammary tumors significantly higher than that of the C3Hf/An. This, however, could represent a genetic difference that may have occurred between the 2 lines.

The incidence of mammary tumors in the substrain C3Hf/An under our breeding system and care was higher than that published earlier by Andervont for the original line.[1] This difference in incidence of tumors may have been due to differences in the systems under which the two colonies were maintained. For instance, our females were fed Derwood pellets and his were fed Purina Lab Chow. Probably more influential, however, was the difference in the system of breeding. While our females were placed with the males at weaning time so that they bred as soon as they matured and continued to have litters as long as they remained fertile, Andervont did not routinely mate his C3H mice during the winter months, and during the other months he routinely mated them at an older age. Another difference that may have contributed to the incidence difference was that he routinely killed any remaining females at 24 months of age, whereas we have permitted the females of our substrains to live their normal span.

Parent-Offspring Correlation

We have concluded that the best test to determine whether a female mouse has the mammary tumor agent is the occurrence of mammary tumors in her susceptible female offspring and in the susceptible females of subsequent generations. Tests of filtrates of mammary tumors are reliable when the results are positive, but when the test females fail to get tumors, the results must be considered indecisive. However, when a pedigree chart is maintained for a susceptible line such as C3Hf, it is found that if a female receives the agent even in a small amount, as when introduced from the male with which she is mated, most of her female offspring will develop mammary tumors at an early age, and the later female descendants will segregate out in the pedigree chart as a high-tumor line.

Pedigree charts were maintained for all four of the substrains reported herein, and in none was there any evidence of high-tumor lines arising in descendants of females with tumors. The females with mammary tumors were scattered throughout all the charts. There was no evidence of an increase or decrease in incidence of tumors in succeeding generations in any of the substrains and no evidence of any lowering of the average tumor age.

The data for each substrain were analyzed further in respect to parent-offspring correlation to ascertain whether or not more tumors occurred in females whose mothers later had tumors than in those whose mothers did not, as would be expected if the tumors in the mothers were the result of the agent. The results of this analysis are shown in TABLE 5. In substrain

TABLE 5
OCCURRENCE OF MAMMARY TUMORS IN FEMALE PROGENY OF TUMOR AND OF NONTUMOR FEMALES

Substrain	Progeny of mothers with tumors		Progeny of mothers without tumors	
	Number	Incidence of tumors (percentage)	Number	Incidence of tumors (percentage)
C3Hf	162	22	577	22
C3Hf/2	36	39	34	65
C3Hff	19	26	73	29
C3Hf/An	19	21	68	29

C3Hf the incidence of mammary tumors in the females whose mothers later had tumors was the same as that in females whose mothers did not have tumors. In the C3Hf/2, C3Hff, and C3Hf/An substrains the incidences in the daughters of mothers without tumors was higher than that of daughters of mothers that later had tumors, although probably not significantly higher except in substrain C3Hf/2. Explanation of this difference is not apparent. Thus, in this parent-offspring correlation analysis there is no evidence of the presence of the mammary tumor agent in the development of these tumors.

The data of substrain C3Hf were next analyzed to see if there was any evidence of a building up of the agent in tumorous females of successive generations. Since the highest number of successive females with tumors was 3, the incidences of mammary tumors were ascertained for females grouped according to whether all of their 3 immediate female ancestors in the direct maternal line of descent had tumors: 2 of the 3 had tumors, 1 of the 3 had a tumor, or none of the 3 had a tumor. Results of this analysis are shown in TABLE 6. There were 6 females whose 3 immediate female ancestors had

TABLE 6
OCCURRENCE OF MAMMARY TUMORS IN C3Hf FEMALES GROUPED ACCORDING TO NUMBER OF ANCESTORS WITH TUMORS

Mother, grandmother, and great grandmother in female line of descent		Number of females	Incidence of mammary tumors (percentage)
No. with tumor	No. without tumor		
3	0	6	0
2	1	51	33
1	2	160	20
0	3	257	23

tumors, and none of these 6 had a tumor. Mammary tumors occurred in 33 per cent of the females with 2 of these 3 ancestors with tumors, in 20 per cent of those with 1 of these 3 ancestors with a tumor, and in 23 per cent of those with none of these 3 ancestors with a tumor. Thus, this analysis failed to give any evidence of the presence of the agent, or its increase in succeeding tumorous females.

Effect of Breeding

The effect of having litters on influencing whether a female will have a mammary tumor has never been very pronounced in our strain C3H mice with the agent. With the high genetic influence and the agent present, the response is so high, being nearly 100 per cent in both breeders and virgins, that the only measurable difference is an increase of from 2 to 3 months in the average age of occurrence of tumors in the virgins over that in the breeders. However, by eliminating the agent the response is brought down to a level at which the effect of the breeding factor becomes more apparent. In TABLE 7 is shown the incidence of mammary tumors in C3Hf females grouped according to the number of litters each had, the highest number being 8. To complete the series, a group of 100 C3Hf females maintained as virgins and reported earlier[8] is also included. These data clearly show that, the more litters a C3Hf female has, the greater is her chance of developing a mammary tumor. The incidences varied from a low of 2 per cent in the virgins to a high of 44 per cent in those that had 7 litters.

TABLE 7
OCCURRENCE OF MAMMARY TUMORS IN C3Hf FEMALES GROUPED ACCORDING TO THE NUMBER OF LITTERS EACH HAD

Number of litters	Number of females	Incidence of mammary tumors (percentage)	Avg. tumor age (months)	Avg. age females died without tumor (months)
0	100	2	22.0	19.9
1	68	13	21.2	20.0
2	127	18	20.2	18.6
3	166	18	20.5	19.2
4	198	24	20.3	17.8
5	180	21	19.2	16.9
6	108	31	19.6	18.3
7	43	44	19.1	19.2
8	9	33	21.0	11.8

Classification of Mammary Tumors

Three hundred and eleven of the mammary tumors occurring in these four substrains were tabulated according to the classification proposed by Dunn (TABLE 8).[11] This number is slightly more than the total number of females

TABLE 8
CLASSIFICATION OF MAMMARY TUMORS OCCURRING IN C3Hf, C3Hff, C3Hf/2, AND C3Hf/An MICE

Type	Number
Adenocarcinoma type A	134
Adenocarcinoma type B	117
Adenocarcinoma type C	1
Adenoacanthoma	53
Carcinosarcoma	6
Total	311

with tumors, since a few had more than one. The adenocarcinomas, predominantly of ascinar structure and classified as type A, were the most numerous, and those in which the cells tended to be in sheets or in chords between cystic spaces, classified as type B, were next most numerous. The tumors in these two groups comprised 81 per cent of the total. In comparison, practically all mammary tumors in our C3H females with the agent can be classified as adenocarcinomas of either type A or type B.

Only one adenocarcinoma type C with small cysts lined with a single layer of cuboidal epithelial cells was found. This type has been found more often in F_1 hybrid females derived from outcrossing C3Hf to C57Bl, and has also been observed in other hybrids.

The adenoacanthomas with a mixture of glandular tumor tissue and epidermoid tissue comprised the third largest group and included 17 per cent of the total number. This type has often been observed in females without the agent, although in these substrains it may not represent a direct effect of the absence of the agent, but rather the result of the condition of the mammary gland due to the advanced age of the females at the time the tumors arose.

Six of the tumors showed a blending of neoplastic epithelial cells and neoplastic spindle cells and were classified as carcinosarcomas.

Other Tumors

Neoplasms other than those of the mammary gland occurring in the females of these agent-free substrains are listed in TABLE 9. The most frequently occurring tumors listed are the hepatomas, which were observed in 13 per cent of the C3Hf females, in 12 per cent of the C3Hff females, in 21 per cent of the C3Hf/2 females, and in 9 per cent of the C3Hf/An females. In substrain C3Hf, with the large number of females, the incidences in specific generations varied from none in generation F_{12} to 37 per cent in generation F_{17}. The usual pulmonary tumor of the mouse occurred in 5 per cent of the females of the C3Hf and C3Hff substrains, in 7 per cent of those of the C3Hf/2 substrain, and in 4 per cent of those of the C3Hf/An substrain.

TABLE 9

NUMBER OF FEMALES WITH TUMORS OTHER THAN MAMMARY GLAND TUMORS

Subline	C3Hf	C3Hff	C3Hf/2	C3Hf/An
Total number of females	911	118	72	96
Hepatomas	117	14	15	9
Pulmonary tumors	41	6	5	4
Tumors of ovary	41	3	8	10
Neoplasms of reticular tissue	23	6	3	6
Tumors of uterus, cervix, or vagina	14	2	2	1
Hemangioendotheliomas	19	1	5	
Subcutaneous sarcomas	17	3	1	3
Squamous cell carcinoma of skin	4			
Squamous cell carcinoma of forestomach		2		
Adenoma of adrenal	2	1		
Osteogenic sarcoma	1			
Sarcoma anterior to kidney	1			
Tumor of harderian gland	2			1

Tumors listed as tumors of the ovary occurred with about the same frequency as did the pulmonary tumors. These tumors of the ovary included tubular adenomas, granulosa cell tumors, and those showing a mixture of cell types. They did not include hemangioendotheliomas found in the ovaries of several females but listed with hemangioendotheliomas occurring elsewhere. The neoplasms listed as those of reticular tissue included lymphocytic neoplasms, Dunn's[12] reticulum cell neoplasms of type A and type B, and some borderline tumors between these types. The tumors of the uterus, cervix, and vagina were sarcomas except for 2 adenomas of the uterus, 2 carcinomas of the uterus, and 1 carcinoma of the cervix. The other tumors occurring in these females are listed in TABLE 9.

These tumors, like the mammary tumors, usually occurred in old females, generally above the average age for the substrain. Few females died at an early age, and the death of most of these was attributable to some cause other than a tumor. Aside from 13 mammary tumors that occurred in females less than 1 year of age, other tumors occurring in females less than 1 year old included only 1 subcutaneous sarcoma, 1 reticulum cell neoplasm, and 1 squamous cell carcinoma of the forestomach.

Summary

Tumors occurring in breeding females of 4 substrains of strain C3H mice deprived of the mammary tumor agent by foster nursing and designated as substrains C3Hf, C3Hff, C3Hf/2, and C3Hf/An are reported. Mammary gland tumors occurred in 22 per cent of the 911 C3Hf females at an average age of 20 months, in 29 per cent of the 118 C3Hff females at an average age of 19 months, in 51 per cent of the 72 C3Hf/2 females at an average age of 20 months, and in 27 per cent of the 96 C3Hf/An females at an average age

of 20 months. In comparison, mammary tumors have occurred in 97 per cent of our C3H breeding females at an average age of 7.8 months.

The females with tumors in these 4 agent-free substrains were scattered throughout the pedigree charts, with no evidence of the segregation of high-tumor lines. Daughters of mothers that died without mammary tumors had an incidence of mammary tumors as high as or higher than those of mothers that later developed mammary tumors. Even as many as 3 successive females with mammary tumors in the immediate maternal ancestry of a female did not increase her chances of developing a mammary tumor.

There was a positive correlation between the number of litters these C3Hf females produced and the incidence of mammary tumors. The incidences ranged from 2 per cent in virgin females to 44 per cent in females that had 7 litters each.

It was concluded that the mammary tumors occurring in these substrains had arisen, in the absence of the mammary tumor agent, as a result of the high genetic susceptibility, the hormonal stimulation from breeding, and the advanced ages of the females.

Of the 311 mammary tumors occurring in these agent-free females, 251 were adenocarcinomas of either type A or type B (the types characteristic of mammary tumors in strain C3H females with the agent); 1 was classified as adenocarcinoma type C; 53 were adenoacanthomas; and 6 were carcinosarcomas.

Tumors other than those of the mammary gland occurring in these agent-free females are listed.

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GENETIC CONCEPTS IN MAMMARY CANCER IN MICE*

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In considering the various aspects of spontaneous mammary cancer in mice, one must be aware of, and constantly keep in mind, the interaction of all the causative factors responsible for the development of the disease(s). Most investigators who have been concerned with the etiology of mammary cancer in mice and have used several inbred strains and hybrids in their studies are of the opinion that there may be more than one "type" of mammary cancer, with perhaps different inciting causes.

In presenting this material it would be possible to review experiments in chronological order, but this would not specify some of the enigmas and pitfalls that may confront the investigator and make the interpretation of the experimental data most difficult. Should any particular question arise, an attempt will be made to consider all pertinent data, rather than to refer to selected observations.

Many general papers have been published on mammary cancer in mice.[33, 37, 40, 50, 71, 74, 83, 86, 105, 109, 113, 121, 132]

In 1939 it was suggested that several causative factors interacted in the development of the usual type of spontaneous mammary cancer in mice, which are:[27-28]

The milk-borne nursing influence with the properties of an infectious agent or virus, now known as the mammary agent (MTA); the inherited susceptibility for spontaneous mammary cancer; and hormonal stimulation of the mammary glands.

The role of the hormones in the development of mammary cancer in mice had been established from the early investigations of several workers.[113] The so-called maternal or extrachromosomal influence was noted following reciprocal crosses between animals of strains having high and low incidences, and where it was observed that the F_1 females with mothers from the cancerous strains had high incidences, while the F_1 hybrids with mothers from the low cancerous strains had low incidences of mammary tumors.[97, 102]

The genetic basis for any inherited character is the percentage of individuals showing the condition in the various generations where segregation takes place. Thus, when one investigates a disease that appears late in life as cancer, it becomes of the utmost importance to have the experimental animals survive to give the highest possible incidence. Furthermore, extrinsic

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factors have been demonstrated to modify the action of some etiological factor(s) and consequently the incidence. These environmental conditions could modify any genetic interpretation of the data.

In 1936[23-29, 63] the maternal influence was found by foster nursing to be transferred in the milk of females of cancerous stocks, and subsequent studies demonstrated that it had the characteristics of an infectious agent or virus. In these and other experiments, it was known by 1939 that hybrid progeny born to females from a strain with a low incidence of mammary cancer, but nursed by females of a cancerous stock, had similar incidences and average cancer ages as did the F_1 offspring born to, and nursed by, mothers of the cancerous strain.[26-29] Cytoplasmic transmission of the maternal influence had been suggested[103, 107-108, 129] and in 1942[75] an intrauterine influence was thought to be of importance. No conclusive data have been obtained to support these theories.[27-29, 64]

The important role played by the genetic susceptibility became evident as soon as the reciprocal F_1 hybrids were nursed by lactating females of the parental strains having extreme incidences of mammary cancer. With this technique, the incidences normally observed in breeding F_1 hybrids could be reversed.[27-29] Other inherited factors also must be given due consideration, especially inherited hormonal patterns, for not only may one be responsible for the induction of the disease in virgin females of susceptible stocks with the MTA,[60-61, 88] but another may inhibit or delay the time of development of mammary cancer in breeding females.[47]

It also was reported that when reciprocal crosses were made between animals of cancerous strains with high incidences, the first generation progeny might not have similar incidences and average cancer ages, even when they were subjected to breeding.[22] Thus, in hybrids where the other etiological causes were assumed to be the same, the milk-borne maternal influence from different cancerous strains need not have the same tumor-inducing activities.

Between 1934 and 1936, data from several crosses were reported when females of the cancerous dilute brown stock were mated with males of other strains.

Little[106] found that the nonyellow offspring derived by crossing dilute brown females with males of the yellow stock had a higher incidence of mammary tumors than did the yellow progeny. The difference extended to animals of the F_2 generation, and it was concluded that the lower incidence in the yellow mice resulted from a reduced hormonal stimulation of the mammary glands, since the yellow mice had a shorter breeding period. Although the incidence of tumors was lower in yellow F_1 hybrids (39 per cent) than in nonyellow (65 per cent), the tumors appeared earlier in the former; the incidence in nonyellow hybrids was higher than was found in breeders of the maternal stock.[125]

Some physiological effects of the yellow gene now are known to be concerned with an increased food intake, causing obesity and eventually sterility,[98] and these effects would account for the difference in the development of mammary cancer in the hybrids considered above.

The outcross between males of the Zavadskaia brachyuric and females o the dilute brown strains produced F_1 females that showed an incidence in virgins of 77 per cent, higher than was found in breeders of the maternal stock. The increased incidence in the F_1 progeny was tentatively explained by Cloudman and Little[67] as resulting from hybrid vigor, with the extra-chromosomal influence considered as the primary causative factor.

Green[97] made reciprocal crosses between animals of *Mus bactrianus* and the dilute brown stocks, and continued the F_1 females as breeders. The incidences in the two groups were 68 per cent and 7 per cent, with the higher incidence being found in hybrids having mothers from the cancerous stock.

A more extensive reciprocal cross was conducted by Murray and Little[97, 128-130] between mice of the dilute brown and C57 black stocks, where the incidences in the F_1 virgin females were found to be 40 and 6 per cent. These data will be given consideration below.

A summary of these studies showed that when females of the same cancerous stock were outcrossed with males of different strains, similar incidences and average cancer ages were not observed in the F_1 progeny, whether they were kept as virgins or breeders. These data would confirm those observed for a cross between two cancerous strains.[22]

The three-factor theory for the development of mammary cancer was advanced in 1939[26-28] after virgin and breeding females of the cancerous A and low cancerous C57 black or B stocks and their reciprocal hybrids had been observed, and after females of several groups had been fostered by females of the two inbred strains. In this study it was possible to compare the incidences of mammary cancer in females subjected to different levels of hormonal stimulation and where some animals had and others were free of the mammary tumor agent. Data were obtained in mice of the hybrid generations on the segregation of genes that controlled the susceptibility for spontaneous mammary cancer. A limited number of mothers was used to secure the hybrids of each generation, and as many progeny as possible were obtained from each female. This gave information on the possible effect that the age of the mothers and the litters in which the young were born had on the subsequent development of mammary cancer. These data have been reported in several publications,[27-29, 32-37] starting in 1937.[63]

A summary of these observations is given in TABLE 1. Because virgin females of the A stock and the hybrids of the A × B (C57 black) cross had low incidences of mammary tumors, whether they had or were free of the MTA,[27-29] only breeders have been tabulated in TABLE 1. Also, breeders *without* the agent were found to have low incidences, and they have not been included, since at this time the data would not contribute to our knowledge concerning the nature of inherited factors for mammary cancer. For example, among nearly 2400 breeders of the agent-free but susceptible Ax and Zb stocks and their reciprocal F_1 hybrids, the incidence of mammary cancer was 0.3 per cent (43), an incidence similar to that observed among 570 breeding females of the C57 black stock by Little, Murray, and Cloudman,[111] or 0.5 per cent.

Data showing that one of the susceptibility genes transmitted by the A stock probably was linked with the gene-producing brown coat color are presented in TABLE 2. Although the observations in the hybrid generations could be interpreted on the basis that the inherited susceptibility was transmitted as a single dominant, it was concluded that multiple factors were involved and that one of these was probably linked with the brown gene.[36]

To illustrate further the difficulty of interpreting the observations obtained in various experiments, it may be of interest to compare the data obtained in three different laboratories when mice descended from the *same inbred strains* were used to produce hybrids and when only virgin females were observed (TABLE 3).

TABLE 1*

DEVELOPMENT OF MAMMARY CANCER IN BREEDING FEMALES OF THE A AND B (C57 BLACK) STOCKS POSSESSING THE MAMMARY TUMOR AGENT†

Stock or generation	No.	Cancer	Av. age in days	
			Cancer	Nonca.
A	972	95%	286	426
B (C57 black)	98	18%	420	518
ABF$_1$ (A♀ × B♂)	123	94%	336	369
BAF$_1$ (B♀ × A♂)	111	90%	346	568
Total F$_1$	234	92%	341	490
ABF$_2$	277	77%	397	535
BAF$_2$	114	69%	452	632
Total F$_2$	391	75%	412	570
ABF$_3$	339	67%	378	545
BAF$_3$	278	66%	388	577
Total F$_3$	617	67%	383	559
Cancerous F$_2$ mothers	508	71%	380	557
Nonca. F$_2$ mothers	109	46%	401	565
ABF$_1$ – BBC (ABF$_1$♀ × B♂)	311	60%	426	626
ABF$_2$ – BBC (ABF$_2$♀ × B♂)	283	51%	437	679
Cancerous F$_2$ mothers	223	63%	435	637
Nonca. F$_2$ mothers	60	5%	480	715

* Reproduced by permission from *Cancer Research*.[35]

† F$_1$ hybrids either nursed their maternal A mothers (ABF$_1$) or the BAF$_1$ hybrids were fostered by females of the A stock; the F$_2$ progeny nursed their mothers, etc.

In virgin females of the cancerous dilute brown stock, the incidences reported were 51 per cent,[128-130] 62 per cent,[127] and 85 per cent,[138] and for those fostered by females of the low cancerous C57 black stock, 9 per cent[138] and 23 per cent.[127] Nonbreeders of the C57 black stock remained noncancerous when nursed by their mothers,[128-130, 138] yet mammary tumors developed in 9 per cent[127] and 13 per cent[138] of the C57 black females nursed by females of the dilute brown stock and kept as virgins.

In the F_1 females resulting from the cross between females of the dilute brown and males of the C57 black stocks, Murray and Little[128-130] observed an incidence of 40 per cent compared with the incidence of 69 per cent reported by Van Gulik and Korteweg;[138] this was reduced to 4 per cent by permitting the hybrids born to the high cancerous females to be nursed by C57 black females.[138] Comparable hybrids were not observed by Murray and Little[128-130] and Murray.[127]

TABLE 2*

MAMMARY CANCER IN THE F_2 HYBRIDS AND BACKCROSS (F_2♀ × B♂) HYBRIDS TO THE LOW CANCER STOCK, DEPENDING UPON COAT COLOR, IN THE CROSS BETWEEN ANIMALS OF THE CANCEROUS A AND LOW CANCEROUS B, OR C57 BLACK STOCKS

Mammary cancer in breeding F_2 hybrids of the A × B cross with the MTA

Color	No.	Cancer	Difference between incidences
Brown	84	88%	Br. and Blk. = 19.2% ± 5.6 or 3.4 × S.E.
Black	209	69%	Br. and Al. = 11.6% ± 5.7 or 2.0 × S.E.
Albino	98	77%	Al. and Blk. = 7.6% ± 5.5 or 1.2 × S.E.
Total	391	75%	

Mammary cancer in ABF_2-BBC hybrids (ABF_2♀ × B♂) and coat color of F_2 mothers

ABF_2 mothers	Ca. F_2 mothers		Nonca. F_2 mothers		Total BBC progeny	
	No.	Cancer	No.	Cancer	No.	Cancer
Brown	110	68%	0	—	110	68%
Black	57	60%	40	0%	97	35%
Albino	56	55%	20	15%	76	45%
Total	223	63%	60	5%	283	51%

* Reproduced by permission from *Cancer Research.*[32, 33, 35, 36, 37]

Low incidences were found in all crosses for the F_1 females that had mothers from the low cancerous C57 black stock, yet when these F_1 hybrids were nursed by females of the cancerous stock, Murray[127] observed an incidence of 65 per cent, contrasted with an incidence of 46 per cent in another study. BdF_1 hybrids nursed by females of the cancerous dilute brown (d) stock were not maintained by Murray and Little.[128-130]

Murray and Little concluded from their observations that the extrachromosomal influence was either six[128] or ten[130] times more important than any chromosomal contribution in the genesis of mammary cancer in mice and that "the tendency to have mammary cancer is not Mendelian in nature."[130] As no reference was made to the reports concerning the nursing influence[23-28, 63] or to the theory on the interaction of the three causative factors[27-28] that appeared prior to their 1939 paper,[130] it must be assumed that they still were of the opinion that the maternal or extrachromosomal influence was cytoplasmic in origin.[107-108, 129] Van Gulik and Korteweg[138] discussed the role of the influence of the milk, genetic, plasma and/or uterus factors in the development of mammary cancer in mice. In a later paper, Murray[127] stated that the ratios in which mammary cancer occurred in the various strains could be explained upon the basis of "the strength of the milk stimulus which the mice receive and the resistance of the physiological systems produced by the matings to various concentrations of the milk stimulus."

TABLE 3*

MAMMARY CANCER DEVELOPMENT IN VIRGIN FEMALES OF THE DILUTE BROWN AND C57 BLACK STOCKS AND THEIR RECIPROCAL F_1 HYBRIDS WHEN NURSED BY FEMALES OF THE PARENTAL STRAINS AND OBSERVED IN DIFFERENT LABORATORIES

Investigation	Stock or hybrid	Nursed by dilute brown females		Nursed by C57 black females	
		No.	Cancer	No.	Cancer
Murray and Little[128-130]	Dilute brown	297	51%	—	—
Van Gulik and Korteweg[138]	Dilute brown	168	85%	110	9%
Murray[127]	Dilute brown	445	62%	108	23%
Murray and Little[128-130]	C57 black	—	—	?	0%
Van Gulik and Korteweg[138]	C57 black	67	13%	194	0%
Murray[127]	C57 black	98	9%	—	—
Murray and Little[128-130]	dBF_1 (dba ♀ × B♂)	113	40%	—	—
Van Gulik and Korteweg[138]	dBF_1	335	69%	47	4%
Murray[127]	dBF_1	—	—	—	—
Murray and Little[128-130]	BdF_1 (B ♀ × dba♂)	—	—	379	6%
Van Gulik and Korteweg[138]	BdF_1	42	46%	722	2%
Murray[127]	BdF_1	93	65%	—	—

* Reproduced by permission from *Cancer Research*.[127, 128, 138]

As discussed above, the MTA transferred by females of different cancerous strains may not have the same tumor-inducing activity in mice with the same genetic constitution,[22] yet when reciprocal F_1 hybrids were nursed by females of the same inbred cancerous stock, they showed similar incidences and average cancer ages.[27-29, 32, 35-37] This also demonstrated that the hybrids that had mothers from the low cancerous strain had the same potentialities for the development of spontaneous mammary cancer when they possessed all the causative factors as did hybrids born to, and nursed by, mothers of the strain with a high incidence of tumors. A summary of experiments in which females of one strain were mated with males of different stocks showed that in these experiments the hybrids did not have the same incidences and average cancer ages.[25] Similar results have been reported by Andervont,[1] Murray,[126] Warner *et al.*,[139] and Murray and Warner.[131] In one study[131] when males of one stock were mated with females of different sublines of a cancerous stock to produce F_1 hybrids, the incidences in the progeny differed by as much as 15 to 76 per cent, depending upon the line to which the mothers belonged. The data obtained from the cross between cancerous animals of the A and C3H strains were influenced, not only by the subline, but by the time when the mice were observed.[45, 48, 59, 61, 88]

Differences in the activity of the mammary tumor agents transferred by mice of either sublines of the same or different cancerous stocks, together with variations in the genetic constitutions of the mice of the separate sublines of the same stock, would account for some of the data presented in the various tables.

TABLE 4

MAMMARY TUMOR IN FEMALES OF SEVERAL INBRED STRAINS AND THEIR RECIPROCAL F_1 FEMALES WHEN MAINTAINED AS VIRGINS

Stock	No.	Cancer	Age	Stock	No.	Cancer	Age
A	111	4%	450	D$_8$	50	66%	459
Z(C3H)	153	66%	404	D$_2$	88	59%	504
A♀Z♂F_1	121	89%	468	Z♀A♂F_1	80	76%	545
A♀D$_8$♂F_1	99	72%	555	Z♀D$_8$♂F_1	111	96%	358
A♀D$_2$♂F_1	67	75%	532	Z♀D$_2$♂F_1	106	94%	351
D$_8$♀A♂F_1	29	38%	598	D$_2$♀A♂F_1	45	24%	680
D$_8$♀Z♂F_1	50	90%	485	D$_2$♀Z♂F_1	83	84%	533
D$_8$♀D$_2$♂F_1	31	67%	546	D$_2$♀D$_8$♂F_1	75	60%	524
A♀I♂F_1	50	72%	467	Z♀I♂F_1	82	99%	324

Observations are presented in TABLE 4 for several reciprocal crosses where the F_1 females were maintained as virgins.

The susceptibility or resistance of animals of inbred strains or hybrid generations to the development of mammary cancer may be determined only after observation of females that have been subjected to, or possess, all the known causative factors responsible for the disease. This involves having them nurse females of strains with high incidences and known to possess the MTA.[27-29] With this technique we propose to test, as described by Little,[109] for the genetic constitution while altering other factors such as hormonal level or other physiological variables. Unfortunately, some strains were "classified" as to their susceptibility or nonsusceptibility before they had been adequately tested, and so the terms tumorous or nontumorous stocks have become synonymous with susceptible or nonsusceptible animals.

One susceptible strain may be low cancerous because of the absence of the agent, another may be low cancerous because the mice are nonsusceptible, while a third, susceptible and with the agent, may be low cancerous because of a lack of hormonal stimulation.

Soon after the maternal influence for mammary cancer in mice became known, Bagg[17] reported that excessive breeding, with the prevention of nursing, would induce mammary cancer in females of the C57 black stock. He referred to this method of breeding as the functional test, or force breeding. Little and Pearsons[112] could not confirm these observations.

Bagg also stated that the C57 black females that developed mammary cancer had been raised by foster mothers[17] and, as determined by personal communication,[33] no record had been kept concerning the ancestry of the foster mothers, as to whether, for example, they possessed the mammary tumor agent. It seems probable that in order to increase the number of animals available for his investigations, Bagg resorted to foster nursing instead of destroying the young and using only C57 black mice nursed by their C57 black mothers. By 1939,[18] Bagg found that normal breeding, with nursing, was as effective as force breeding in the genesis of tumors. He determined[19] that 47 females of his low C57 black subline remained noncancerous after being subjected to the functional test, but when 18 females of the same subline were nursed by females of the cancerous C3H stock and presumably continued as normal breeders, when the report was submitted 50 per cent had developed mammary cancer and 33 per cent were still living.

Andervont[4] found that while the descendants of the animals obtained from Bagg had a low incidence of mammary cancer, mammary cancer developed in 63 per cent of the C57 black females that had been fostered by females of his cancerous C3H stock.

Selected data obtained by various investigators for C57 black females that had been fostered by females of cancerous strains are recorded in TABLE 5.

TABLE 5

SELECTED MAMMARY CANCER OBSERVATIONS IN C57 BLACK FEMALES FOSTERED BY FEMALES OF CANCEROUS STOCKS AND, IN SOME CASES, THEIR PROGENY

Fostered by	No.	Cancer	Av. age (months)	Source
Strain A	8	38%	14.1	Bittner, 1937[24]
Strain C3H	14	14%	5.7	Andervont, 1940[2]
Strain A	104	11%	13.2	Bittner, 1940[29]
Strain dba	67*	13%	—	Van Gulik and Korteweg, 1940[138]
Strain dba	98*	9%	—	Murray, 1941[127]
Strain dba	10†	50%	—	Fekete and Little, 1942[75]
and their descendants	93	73%	—	
Strain C3H	35	63%	14.7	Andervont, 1943[4]
Strain A	98	18%	13.0	Bittner, 1944[36]
Strain RIII	92	76%	15.8	Haagensen and Randall, 1945[81]
and their descendants	100	69%	13.7	
Strain C3H	50	0%	—	Andervont and Dunn, 1948[10]

* All maintained as breeders except two groups maintained as virgins.
† One group descended from transferred ova.

Animals free of the MTA have not been included since only an occasional tumor will be found,[111] comparable in incidence to that found in most agent-free susceptible strains.[34, 37, 43]

In only one study, completed by Andervont and Dunn,[10] did breeders of the C57 black stock fail to have mammary tumors when they had the MTA. Two females of another fostered litter died noncancerous, but 1 of 9 of the C57 black progeny had the disease.[5] Because of the low incidence, a few mice were obtained from Andervont, and this subline of the C57 black stock has been maintained in our colony for several years. In 1 litter of 3 C57 black females nursed by a member of our Z(C3H) cancerous stock, mammary tumors developed in 3 of their progeny at an average age of 408 days, an incidence of 20 per cent.[50] Cancerous females have not been observed in other fostered litters and their C57 black descendants of this subline.

In other experiments where females of the C3H cancerous stock were used as the foster mothers of C57 black mice obtained from different sources, Andervont[2, 4] observed incidences of 14 per cent and 63 per cent. When females of the cancerous RIII stock were the nursing females, Haagensen and Randall[81] found that 76 per cent of the C57 black females of the fostered generation and 69 per cent of their progeny had mammary cancer.

Fekete and Little[75] transferred fertilized C57 black ova to the uteri of females of the dilute brown cancerous stock. Of the C57 black females which had progeny after nursing their dilute brown "mothers," from whom they would obtain the MTA, 50 per cent had mammary cancer (the few which did not have offspring remained noncancerous). The progeny and descendants of transferred-ova C57 black females were continued for three generations; although these black mice developed in the uteri of C57 black mothers, nevertheless 73 per cent developed mammary tumors. No biological assay was made to determine the propagation and transmission of the mammary tumor agent in the study.

The following citations concerning the history of the C57 stock may offer some explanation why some workers found a high incidence of mammary tumors in some groups while others did not (TABLE 5).

Little and Bagg[110] obtained black mice that gave rise to the C57 black stock from Miss Lathrop, but the offspring of these animals were heterozygous for several coat color genes. Pedigree charts given by Little and Bagg[110] and Murray[123] showed that the C57 black and C57 brown sublines had a common ancestry, descended from the original pair of black animals, No. 57 ♀ × No. 52 ♂. In 1925, when the C57 brown line came under the supervision of J. M. Murray,[123] it had been continued for eleven generations but, as late as the fourteenth generation, matings were made between animals of sublines that had been separated for several generations.

In 1939 Little, Murray, and Cloudman[111] reported on the occurrence of nonepithelial tumors in mice of the C57 black stock and tabulated the tumors according to the subline to which the mice belonged. Of the several lines included, only two animals of one line were cancerous, while 76 of another line died of tumors. Three mammary tumors were observed among the C57 black breeders, an incidence of 0.5 per cent. These sublines within the

C57 black stock traced back to single pairs brought from Ann Arbor, Mich. to the Jackson Memorial Laboratory, Bar Harbor, Me., in 1929.[111]

The observations on the development of mammary cancer in C57 black mice that carried the agent and were used as breeders would support the conclusion that animals of the C57 black stock became known as being relatively resistant or nonsusceptible for the disease before they had been adequately tested. The material presented in TABLE 5 would demonstrate that mice of some sublines of the C57 black stock, with incidences in excess of 60 per cent,[4, 18, 19, 75, 81] should be considered as "susceptible" for the development of mammary cancer as are members of some so-called cancerous-susceptible stocks. In addition, these data would support the observations made by Little, Murray, and Cloudman[111] to the effect that, just as there were sublines within the C57 black stock that differed in their susceptibilities for the development of nonepithelial tumors, so do these lines differ in their susceptibilities for spontaneous mammary cancer. This became evident only after the mice had been observed as breeders and they had obtained the MTA by nursing females of cancerous strains.

The dilute brown stock was separated into sublines about 1927[104] and it has been found that tumors that develop in mice of one line may not grow progressively when transplanted in members of other lines.[40, 66, 104] While genetic differences would be involved, they may or may not influence the factors that determine the development of spontaneous mammary cancer,[40] although the controls for any study should be representatives of the same subline. Likewise, mice from several lines should not be tabulated together to give a larger number of individuals.

Representative data for the development of mammary tumors in the dilute brown stock are recorded in TABLE 6.

As with the C57 black stock, the influence of force breeding has not been definitely established for the dilute brown stock, either for mice with or without the agent. Mühlbock[117, 122] presented evidence that force breeding increased the incidence and caused the tumors to appear earlier, yet virgin dilute brown females observed in another series[118] showed an earlier cancer age and higher incidence than did females that cast two litters and were not permitted to nurse their progeny. In this laboratory the functional test of Bagg, or force breeding, neither increased the incidence nor accelerated the time of development of cancer in females of three cancerous sublines.[40]

Between 1946 and 1951 the incidence of mammary tumors remained relatively constant in breeders of the D_8 subline but, after another group of D_8 mice had been nursed by a female of the cancerous Z(C3H) stock, these fostered females and their descendants of the three succeeding generations had higher incidences than did the unfostered dilute brown mice (TABLE 6, unpublished data). Only a difference of ten days was seen between the average cancer ages in the breeders and virgins of the fostered group of D_8 females with the MTA of the Z stock, and 88 per cent of the nonbreeders died cancerous.

With the exception of those observed by Mühlbock *et al.*,[122] low incidences have been observed for agent-free females of the dilute brown stock when they

TABLE 6

OBSERVATIONS MADE BY DIFFERENT INVESTIGATORS ON THE DEVELOPMENT OF MAMMARY CANCER IN FEMALES OF THE CANCEROUS DILUTE BROWN STOCK

Subline	Observed as	No.	Cancer	Ca. age	Investigation
?	Breeders	479	77%	10.5 mo.	Murray, 1928[124]
	Virgins	207	12%	15.8 mo.	
?	Breeders	2251	59%	10.6 mo.	Murray, 1934[125]
?	Virgins	297	51%	?	Murray and Little, 1935[128]
?	Breeders	80	76%	12.7 mo.	Korteweg, 1936[103]
	Virgins	197	72%	13.4 mo.	
?	Virgins	168	85%	?	Van Gulik and Korteweg, 1940[138]
?	Virgins	445	62%	?	Murray, 1941[127]
A	Breeders	19	63%	418 days	Burrows, 1941[65]
B	Breeders	132	58%	498 days	
C	Breeders	134	57%	441 days	
D	Breeders	92	57%	442 days	
E	Breeders	69	10%	439 days	
D_1	Virgins	45	51%	504 days	Bittner, 1948[40]
	Breeders	50	82%	366 days	
	Fr. breed.	24	67%	451 days	
D_2	Virgins	88	59%	504 days	Bittner
	Breeders	134	69%	387 days	
	Fr. breed.	34	56%	403 days	
D_8	Virgins	50	66%	459 days	Bittner
	Breeders	62	77%	405 days	
	Fr. breed	19	63%	399 days	
DBA/2	Breeders	?	43%	15 mo.	Andervont and Dunn, 1948[11]
DBA/1	Breeders	60	38%	11.7 mo.	Hummel and Little, 1949[93]
?	Virgins	86	59%	546 days	Mühlbock, 1950[117]
	Fr. Br., 2 lit.	48	62%	477 days	
	Fr. Br., 3+ lit.	69	97%	382 days	
?	Virgins	145	70%	450 days	Mühlbock, 1951[118]
	Breeders	198	69%	417 days	
?	Virgins	86	59%	546 days	Mühlbock *et al.*, 1952[122]
	Breeders	151	68%	408 days	(3 or more litters, av. of 6)
	Fr. breed.	48	62%	477 days	(av. 2 litters)
	Fr. breed.	69	97%	382 days	(av. 7 litters)
DBA/1	Breeders	141	72%	356 days	Chai and Russell, 1956[66]
DBA/2	Breeders	183	56%	401 days	
Others	Breeders	193	65%	390 days	
Total	Breeders	517	64%	383 days	
D_2	Breeders	165	76%	386 days	Bittner (unpublished)
D_8	Breeders	186	77%	459 days	
D_8z	Virgins	51	88%	435 days	Bittner (unpublished)
D_8z	Breeders	54	93%	425 days	

were used as breeders.[13, 100, 122] Because of the subline differences found by Mühlbock *et al.*,[122] the slight variation found between normal and force breeders could have resulted from including unequal numbers of mice from the various lines in the two groups.

Several reports have been made upon the development of mammary cancer in dilute brown mice descended from transferred ova.[63, 76, 93] Fekete and Little[76] observed an incidence of 12 per cent among the descendants, while in those continued by Hummel and Little[93] only 3 per cent of the descendants continued for 25 generations had cancer. It previously was pointed out[37] that 23 per cent of the descendants of 6 transferred-ova dilute brown females had mammary cancer in the group studied by Fekete and Little,[76] while the descendants of 13 other transferred-ova mothers remained noncancerous. This difference probably was significant, and tumors were not assayed for the MTA.

Representatives of the C3H stock[137] have been observed in many experiments, and representative data on mammary cancer are tabulated in TABLE 7. Again, subline differences have been encountered.[3, 37]

Mice of the Heston C3H line[92] were separated from the Andervont C3H colony in 1941; whereas Andervont and Dunn[11] observed an incidence of only 4 per cent in agent-free breeders, Heston *et al.*[92] found mammary cancer

Table 7

Observations on the Development of Mammary Cancer in Females of the Cancerous C3H Stock, Tabulated by Sublines: Andervont (HBA), Heston (WEH), and Bittner (JJB). Average Cancer Ages in Days

Subline	Observed as	No.	Cancer	Ca. age	Investigation
HBA	Breeders	1588	91%	258	Andervont, 1941[3]
	Virgins	350	97%	312	
HBA	Breeders	1553	92%	243	Andervont, 1949[6]
	Virgins	346	93%	339	(F_{28}–F_{46})
WEH	Breeders	419	97%	234	Heston *et al.*, 1950[92]
	Virgins	46	100%	318	
WEH	Breeders	155	93%	255	Boot and Mühlbock, 1956[64]
	Fr. Breed.	28	82%	209	
	Virgins	117	79%	305	
HBA	Breeders	136	91%	228	Bittner, 1956[52]
	Virgins	112	93%	348	
JJB	Breeders	200	78%	321	Bittner, 1935[20]
JJB	Breeders	227	89%	336	F_{23}–F_{36}, Bittner, 1943[34]
	Breeders	164	90%	303	F_{37}–F_{44}
	Breeders	214	97%	267	F_{45}–F_{51}
JJB	Breeders	224	95%	273	F_{52}–F_{57}, Bittner and Huseby, 1946[59]
	Virgins	92	63%	399	
JJB	Breeders	54	94%	272	Bittner, 1948[40]
	Fr. Breed.	30	80%	310	
	Breed.	87	93%	270	
	Nursed 12 days	47	74%	263	
	Fr. Breed.	48	81%	234	
JJB	Breeders	599	97%	272	Bittner, 1956[52]
	Virgins	54	87%	386	
JJB, fost. by A	Breeders	80	93%	321	Bittner, 1956[52]
	Virgins	78	72%	397	

in 38 per cent of the breeders and 4 per cent of the nonbreeders without the MTA. The average cancer age was in excess of 20 months and the agent could not be demonstrated in the tumors by biological assay.[92] The high incidence seen in agent-free females of the Heston C3H subline was confirmed by Boot and Mühlbock,[64] since more than 40 per cent of the normal breeders and over 50 per cent of the force breeders had the disease, and the incidence could be increased by the subcutaneous transplantation of pituitary grafts.[64, 12] Boot and Mühlbock[64] observed mice descended from transferred-ova, but were unable to find any evidence for any intrauterine influence in the genesis of the tumors.

The incidence of mammary cancer in breeding females of our agent-free C3H or Zb stock, fostered in 1938, has remained low, and less than 1 per cent have developed mammary cancer.[34, 43, 59]

The development of mammary cancer in females of our C3H line may be influenced by permitting the mice to nurse females of another cancerous stock having a MTA with a different tumor-inducing activity.[40]

In 1935,[21] following a change in diet, the incidence of mammary cancer in breeding females of the A stock increased to over 80 per cent and has remained quite constant,[27, 36, 43] except for those maintained during the war period,[59] when it became necessary to make substitutions in the animal food. Hummel and Little[93] reported in 1949 that only 54 per cent of the breeding females of their strain died cancerous. In most experiments[26, 27, 59, 93] not more than 5 per cent of the nonbreeding females of the A stock had tumors, the exceptions being those observed by Warner *et al.*[139]

In agent-free Ax breeding females descended from a member of the first litter to be fostered in 1934,[23] the incidence for females born between 1943 and 1948 was 0.3 per cent.[43] Tumors which arise in mice of the cancerous A stock have been found to grow progressively, when transplanted, in mice of the Ax agent-free line.

The MTA may be recovered from many tissues of infected mice,[37] including seminal vesicles[10] and cauda epididymidis.[116] During the past ten years numerous experiments have demonstrated that males of cancerous strains with the MTA may infect agent-free females at coitus[7, 10-12, 15, 41, 43, 50, 52, 54, 55, 70, 77, 91, 116, 117, 119] and, after this occurs, the agent may be demonstrated by biological assay in the mammary tumors from infected females and their progeny. Agent-free females with amputated uterine horns, but with intact ovaries, may become infected,[58] and vasectomized males of cancerous strains also may transfer the agent.[54-55]

Females of strains free of the agent differ in their sensitivity to become infected, and males of cancerous strains vary in their ability to infect agent-free females.[15, 43] By fostering males of a cancerous strain on females of another cancerous stock, so that they obtain a different MTA, it is possible to alter the capability of the males to infect females.[50, 58]

Studies completed by several investigators[41, 43, 50, 52, 54, 55, 58, 91, 119] have demonstrated that, as soon as the females became infected from the male, they will pass the agent to their progeny in the milk, regardless of whether the mothers died noncancerous or had mammary cancer. Among the progeny born to infected mothers the incidence and average cancer age were found to be similar for those born in the first, as in later litters.[54] In most crosses, the offspring born to these females before they were infected had a low incidence and the cancer age may have been in excess of 20 months; also, the MTA could not be demonstrated in these tumors.

Andervont and Dunn[5, 6, 10-12, 15, 16] found that a considerable proportion of F_1 hybrids obtained by crossing animals of their C3H and Bagg albino stocks had mammary tumors, and that the agent could not be demonstrated except from tumors that occurred in young animals and where most of the littermates died cancerous. In one experiment, no significant difference was noted between the incidences among the hybrids sired by C3H males with and without the agent.[15]

These data have been corroborated in this laboratory by using animals of the same stocks.[52] The average cancer age was in excess of 20 months; when only noncancerous hybrids were included that survived for 600 days, the incidence in the F_1 hybrids was 51 per cent. The incidence was the same for the offspring born in the first two litters as in later litters to the same mothers, and the biological assay studies for the agent were negative.

Another series of hybrids is being observed; in this group some males of the C3H/AnBi stock did infect some C females, and the tumor-inducing activity of the agent recovered from these tumors was as great as any ever tested in any previous assay.[54, 55] It also has been shown that the ability of males of the C3H/AnBi cancerous stock to infect agent-free females may be enhanced by nursing the mice on females of our cancerous Z(C3H) stock. The hybrids born to females infected by these males behave like those sired by males of our C3H stock following infection, and the biological tests are as active.

The mammary tumor agent may "appear" suddenly in mice that have never been nursed by females with the agent, nor injected with extracts, nor mated with males of cancerous stocks.[30, 40, 43, 72, 73] Should this occur in mice that are susceptible, lines with high incidences of tumors may be developed.[30, 43]

Of necessity, certain statements concerning the propagation and transmission of the MTA must be associated with the development of cancer in strains having a low incidence; perhaps these were considered previously. Andervont[5] found that, while two C57 black females of one litter nursed by a female of the cancerous C3H stock remained noncancerous, one of their nine progeny had cancer. Although the females of the fostered generation transferred the agent, none of their progeny, including the female that developed cancer, did so.

Heston, Deringer, and Andervont[90] concluded from the incidences noted in two groups of backcross hybrids that nursed F_1 females, obtained by mating animals of the C3H and C57 black stocks, that multiple factors determined the propagation and transmission of the agent. In a more recent study, Heston, Deringer, and Dunn[91] found that the agent had been eliminated by the third backcross generation to the resistant strain, and this would suggest the possibility that the two inbred strains differed by only one gene that controlled the agent. If one generation must lapse before the agent is lost in the absence of the gene, the data obtained in the earlier study[90] also could be explained according to this single-gene theory.

Animals of the same subline of the C57 black stock were obtained from Andervont, and some of their descendants have been nursed by females of our cancerous Z(C3H) stock. Thirteen C57 black females were fostered and died noncancerous, but mammary cancer developed in 4 of their 32 progeny, 2 born in the same litter.[50] Four progeny born in the second litter to 1 fostered female remained noncancerous and only 1 propagated the agent, but the two C57 black offspring born in the third litter developed mammary cancer and both transferred the agent. Since we employed C57 black mice with the same genetic make-up as others,[5, 90, 91] the data would indicate that different "agents" had been obtained from the fostering mothers. These agents were not propagated and transmitted with the same effectiveness by mice of an inbred, resistant subline of the C57 black stock. Also, differences may exist between mice born in the same litter.

Fekete and Little[75] presented evidence that the MTA may survive for at least four generations, or as long as the line was continued, in females of the C57 black stock. In their study, fertilized ova from C57 black females were transferred to the uteri of females of the dilute brown stock, and the "C57" mice that developed then nursed their dilute brown "mothers," from whom they would obtain the mammary tumor agent. Ten of these C57 black females had progeny, of which 5, or 50 per cent, had mammary cancer, as did 73 per cent of their descendants of the succeeding 3 generations, although the latter developed in the uteri of black females. If the C57 black females of the "fostered" generation had mammary cancer, the incidence in their descendants for 3 generations was 91 per cent, compared with 62 per cent for those descended from fostered females which died noncancerous.

Females of another subline of the C57 black stock may propagate and transmit the mammary tumor agent for many generations, and Dmochowski[72, 73] also determined that 7 to 9 generations may intervene in the line between cancerous animals. The incidence of mammary tumors in breeders of this C57 subline was 15 per cent, and the agent could be recovered from the tumors by biological assay, yet 75 females of the same generations remained noncancerous when they were continued as nonbreeders or virgins. Thus, one could conclude from observing virgin females that the MTA was not present, since they did not have mammary cancer; nevertheless, by subjecting females to the hormonal stimulation of breeding and lactation, mammary tumors appeared and the MTA could be demonstrated in mice of this C57 black line of mice.

In 1940,[19] Bagg stated that between 76 and 89 per cent of the members of his cancerous line of C57 black had mammary cancer. Based upon our present knowledge, presumably this would imply that the line had remained high cancerous for at least twelve generations and that the agent, obtained from foster mothers[33] had been propagated through successive generations of C57 black mice.

If mammary tumors are transplanted that develop in C57 black females that possess the agent, the agent could be demonstrated in the tumors after they were carried for ten[39, 46] and 42[69] passages in C57 black animals that themselves did not have the agent.

Andervont[8] and Andervont and Dunn[16] described a milk-borne tumor-inducing agent to be transferred for many generations in a line of wild mice, although the wild mice had a low incidence of mammary cancer. However, a considerable proportion of agent-free susceptible mice developed mammary cancer after being nursed by females of the wild strain. Thus, the wild mice were resistant to the action of the agent that they were able to propagate and transfer, and that would produce tumors in domestic mice.

Starting with F_1 females obtained by mating females of the cancerous dilute brown stock and males of the C57 black stocks, and backcrossing to males of the C57 stock for eight generations, Murray and Little[130] reported that with the concentration of the C57 black chromatin the extrachromosomal influence became noneffective. They concluded that the extrachromosomal influence was ten times more important than any possible chromosomal influence in the genesis of mammary cancer in mice, and that "the tendency to have mammary cancer is not Mendelian in nature." Since no reference was made to either the nursing influence[23, 24] or the reports on the action of several causative factors in the development of mammary cancer[27, 28] that appeared prior to their publication,[130] one may assume that their "extra-chromosomal influence" was still considered to be dependent upon the source of the cytoplasm.[129] As such, it need not be included with studies on the genetic control of the propagation and transmission of the milkborne MTA.

Murray and Little[128, 130] did not observe the development of tumors in hybrid females that had been maintained as breeders and, after eight generations of backcrossing to the low cancerous stock, the hybrids would have a genetic constitution similar to C57 black mice. It has been established by Fekete and Little,[75] Dmochowski,[72, 73] and Boot and Mühlbock[64] that C57 black females possessing the MTA may not have mammary cancer unless they are permitted to have progeny.

Low incidences of mammary cancer have been observed in breeding females of the I stock after they have secured the MTA by nursing females of cancerous stocks.[2, 4, 45, 50] Again, it was noted that the strain of foster mothers influenced the propagation of the agent and, even in the same litter, one female might transfer the agent while another might not.

Until 1939[113] it was believed that only a small proportion of ovariectomized females of cancerous strains would develop mammary cancer and that, the younger the mice were when the ovaries were excised, the lower the incidence would be. Then Woolley and his associates[76, 141-150] reported upon the influence of gonadectomy in one-day-old mice. In two groups of dilute brown females, mammary tumors developed in 27 per cent[145-147] and 49 per cent[76] of the ovariectomized females. The adrenal cortex had become hyperplastic in the castrated animals and there was hormonal stimulation of the secondary sex organs. Mice of some stocks remained in the castrate state.

An analysis of the data revealed that ovariectomized females of cancerous strains that *had high incidences of mammary tumors as virgins also developed postcastrational adrenal cortical lesions.*

To study this possible relationship, a cross was made between animals of the A and Z(C3H) strains, because of their respective low and high incidences in nonbreeders, and mice were provided to enable Smith[133-135] to investigate the influence of ovariectomy. Mammary tumors developed in the reciprocal F_1 intact hybrids, whether they were continued as breeders or virgins, demonstrating that the hormonal mechanism responsible for the induction of the disease in virgins was transmitted;[61] similar results were reported by Heston and Andervont[88] almost concurrently. Adrenal cortical alterations were found in the ovariectomized females that had mammary cancer and were representatives of groups of nonbreeders that would have had mammary cancer, while the females of the cancerous-susceptible A strain remained in the castrate state.[133-135] The virgin females of the A stock had been shown to have a low incidence of mammary tumors.[26-28]

With this preliminary information, mice of inbred strains were tested for their intrinsic capacity to develop adrenal lesions following gonadectomy, and these stocks were then used as parents of hybrids to study the physiological effects of their inherited hormonal mechanisms upon the genesis of mammary cancer.[59, 60, 94-96]

After many inbred strains and hybrids had been observed, the transmission of the adrenal lesions following castration was summarized by Huseby and Bittner[95] as follows: "The type of adrenal alteration was inherited equally through male and female parents and the tendency to become carcinomatous was dominant over that for hyperplasia to develop, but . . . both of these were dominant over the tendency for no marked cortical alteration to appear."

The observations on the development of mammary cancer in virgin F_1 hybrids derived by mating females of the cancerous A stock with males of different stocks are tabulated in TABLE 8. The maternal parents were used because of the low incidence of mammary cancer in virgin females,[27-29, 59] and because the ovariectomized females of the A stock remained in the castrate state.[133-135] The paternal strains were tested because, in most instances, it had been shown that gonadectomized animals would develop postcastrational adrenal cortical hyperplasia and/or carcinoma.[50]

As may be seen from the data, virgin females of the inbred A stock and hybrids of the ABF_1 generation (A ♀ × C57 black ♂) had similar incidences of mammary cancer;[27-29] ovariectomized females of the inbred parental strains show little, if any, adrenal lesions and hormonal stimulation.[133-135, 146] However, ABF_1 hybrids have a high incidence as breeders, as do females of the maternal A stock;[27-29, 35-37] the incidence in C57 black mice is dependent upon the subline being tested (TABLE 5).

In seven other groups of F_1 hybrids, instead of an incidence of 5 per cent characteristic of the maternal stock, the incidence of mammary tumors was in excess of 65 per cent (TABLE 8), and in every case the gonadectomized animals of the *paternal* strains gave rise to adrenal changes. Included were mice of the I stock that are resistant to the development of spontaneous mammary cancer after they obtain the MTA.[1, 4, 45, 60, 94] Members of the so-called I^s line, obtained from Strong[137] in 1950, likewise are nonsusceptible for the disease (unpublished data). Data are not complete on the transmission of the hormonal pattern by mice of the C(BALB/c) and CBA strains, demonstrating that they transmit the inducing mechanism. The mammary cancer-inducing hormonal pattern was called the inherited hormonal influence[37] but, as other strains were studied, it has become evident that there probably are several inherited hormonal mechanisms, one of which may "inhibit" mammary cancer in breeding females and give a reduced incidence.[47, 50-51]

Table 8

Mammary Cancer in Virgin F_1 Females Having Mothers from the A Stock

Hybrid	Mating	No.	Cancer	Average age in days	
				Cancer	Nonca.
AZF_1	A♀ × C3H♂	121	89%	468	601
$ACBAF_1$	A♀ × CBA♂	45	78%	530	535
ACF_1	A♀ × C♂	51	75%	474	671
AD_2F_1	A♀ × D_2♂	67	75%	532	675
AD_8F_1	A♀ × D_8♂	99	72%	555	724
AI^bF_1	A♀ × I^b♂	50	72%	451	681
AI^sF_1	A♀ × I^s♂	82	65%	438	707
$ACEF_1$	A♀ × CE♂	71	37%	568	701
$ANHF_1$	A♀ × NH♂	128	16%	567	689
$AJKF_1$	A♀ × JK♂	115	17%	651	725
ABF_1	A♀ × B♂	125	5%	657	801
A	A♂	111	4%	450	573

Future publications, in collaboration with R. A. Huseby, will consider the development of mammary cancer in gonadectomized male and female mice of groups not previously discussed. In some, no difference was found between the sexes, and at least 80 per cent of all mice had mammary cancer; in others, the ovariectomized females had a high and the castrated males a low incidence while, in still other series, few of the gonadectomized males and females had the disease. In the mice showing these differences in the genesis of mammary tumors, there might not be any difference, as far as could be determined, in the inherited susceptibility for mammary cancer, the activity of the MTA, and the appearance of the postcastrational adrenal and/or pituitary lesions.

Although the gonadectomized animals of the CE,[78, 79, 99, 148-150] NH,[47, 50, 51] and JK[50, 95] (Huseby and Bittner, unpublished data) strains show adrenal cortical lesions, their hormonal mechanisms will not induce a high incidence of mammary cancer in nonbreeding susceptible mice with the agent. That s, after males of these strains were mated with females of the A stock, their virgin F$_1$ progeny did not have incidences of mammary tumors typical of the action of the mammary cancer-inherited hormonal influence. However, when females of the Z(C3H) strain, with the inducing hormonal mechanism, were used as the maternal parents to produce the F$_1$ hybrids instead of females of the A strain, their progeny had high incidences. Some effects of the hormonal patterns transmitted by the parental stocks may be evident in determining the average cancer ages. These data are given in TABLES 9 to 11, together with the observations for hybrids with parents of the I stock (TABLE 9).

TABLE 9*

DATA ON THE DEVELOPMENT OF MAMMARY CANCER IN INBRED MICE OF THE A, Z(C3H), AND I STOCKS AND THEIR HYBRIDS

Stock or generation	Matings	Observations for breeding females				Observations for virgin females			
		No.	Per cent cancer	Av. age in days		No.	Per cent cancer	Av. age in days	
				Cancer	Nonca.			Cancer	Nonca.
A	A♀ × A♂	569	84	330	387	111	4	450	573
Z(C3H)	Z♀ × Z♂	858	95	278	322	153	66	404	499
I†	I♀ × I♂	53	0		463	0			
AZF$_1$	A♀ × Z♂	85	94	320	414	121	89	468	601
AZF$_2$	AZF$_1$♀ × AZF$_1$♂	116	83	337	367	153	62	468	683
AZF$_1$-ABC$_1$	AZF$_1$♀ × A♂	22	91	328	626	22	41	500	706
AZF$_1$-ABC$_2$	AZF$_1$-ABC$_1$♀ × A♂	19	89	389	549	159	19	498	640
AIF$_1$	A♀ × I♂	97	95	224	389	50	72	451	681
AIF$_2$	AIF$_1$♀ × AIF$_1$♂	82	68	302	549	155	30	469	676
AIF$_1$-ABC$_1$	AIF$_1$♀ × A♂	23	78	314	609	80	16	540	681
AIF$_1$-IBC$_1$	AIF$_1$♀ × I♂	25	56	274	631	84	44	482	715

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† Eleven females of the I stock, fostered by Z or A females, and 42 descendants.

Of special interest in the cross between females of the A and males of the I stocks was the high incidence of mammary tumors to be observed in the AIF$_1$ virgin females, demonstrating that mice of a nonsusceptible strain may transmit the hormonal influence that will induce mammary cancer in susceptible mice with the MTA. Also, it became evident that the same genes did not control the effects of the hormonal pattern that induces mammary cancer and the inherited susceptibility for the development of spontaneous mammary cancer, although genes common to both may exist (TABLE 9).

In the hybrids produced by mating females of the A stock with males of the susceptible Z(C3H) and nonsusceptible I stocks, it was possible to compare the influence of the hormonal patterns of the parental strains in the induction of mammary cancer, perhaps to secure some information concerning their physiological effects and, from the percentage of the hybrids which had mammary cancer, to estimate the number of genes involved in producing

TABLE 10*

OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN HYBRIDS, DERIVED BY CROSSING MICE OF THE A, Z(C3H), AND CE STOCKS

Hybrids	Matings (♀ × ♂)	Observed as breeders				Observed as nonbreeders			
		No.	Cancer (per cent)	Av. age		No.	Cancer (per cent)	Av. age	
				Cancer	Non-cancer			Cancer	Non-cancer
				(days)				(days)	
ZCEF$_1$	Z × CE	71	95	273	361	79	95	402	591
ZCEF$_2$	ZCEF$_1$ × ZCEF$_1$	21	81	314	366	22	82	463	561
ZCEF$_1$-AxBC	ZCEF$_1$ × Ax	18	100	268		31	52	577	768
ZCEF$_1$-ZbBC	ZCEF$_1$ × Zb	14	93	292	322	25	88	406	599
ACEF$_1$	A × CE	54	91	341	537	71	37	568	701
ACEF$_2$	ACEF$_1$ × ACEF$_1$	52	65	477	626	92	17	572	700
ACEF$_1$-AxBC	ACEF$_1$ × Ax	12	83	213	599	24	13	481	708
ACEF$_1$-ZbBC	ACEF$_1$ × Zb	12	100	327		34	91	418	594
A†	A♀ × A♂	569	84	330	387	111	4	450	573
Z(C3H)†	Z♀ × Z♂	858	95	278	322	153	66	404	499
CEz (fost.)‡	CE♀ × CE♂	123	83	456	594	34	26	611	718

* Reproduced by permission of *Cancer Research*.[49]
† These data from *Cancer Research*.[47]
‡ These data also from *Cancer Research*.[57]

TABLE 11

MAMMARY TUMORS OBSERVED IN VARIOUS GROUPS OF MICE, ALL HAVING THE MTA

Stock	Observed as virgins			Observed as breeders		
	No.	Ca.	Age	No.	Ca.	Age
CEz (fost.)*	34	26%	611	123	83%	456
CEz	—			19	89%	362
CEzI^{b}F$_1$	50	88%	377	31	97%	311
CEzI^{a}F$_1$	46	85%	359	19	95%	287
CEzJKF$_1$	36	36%	538	22	59%	353
AJKF$_1$	115	17%	651	74	43%	475
ZJKF$_1$	38	84%	415	15	93%	257
AIF$_1$	50	72%	451	97	95%	224

* Fostered in earlier series.

he hormonal effects and also the inherited susceptibility for mammary ancer.

In comparing the A♀ × Z♂ and A♀ × I♂ hybrids it was observed hat, while inducing hormonal patterns were transmitted by both male trains, the incidences of mammary tumors in the hybrids were not in accord vith the theory that the same genic make-up was active in the two strains, lthough the physiological effects might be similar. Further evidence of he segregation of the genes associated with the two inherited factors—suseptibility and hormonal—may be seen in the F_2 and back-cross generations TABLE 9).[45] No explanation may be suggested for the acceleration in the ime of appearance of tumors in the hybrids possessing chromatin from the I tock, contrasted with the corresponding hybrids sired by males from the ancerous Z(C3H) stock.

The incidences of mammary tumors are compared in TABLE 10 for females f the A, Z(C3H), and CE stocks when they possess the MTA and were coninued as either virgins or breeders. At least 80 per cent of the CE breeders hat had two or more litters had mammary cancer, but the incidence was nly 26 per cent in nonbreeding CE females with the agent.[49, 50, 57]

The hybrids with mothers of the Z(C3H) stock had high incidences, vhether they were permitted to have progeny or were kept as virgins, but sually only the breeding hybrids with maternal parents from the A stock ad a high incidence. Data are given for the hybrids of the F_2 generations nd other generations produced by mating the F_1 females with males of the A and Z strains, using agent-free males to control male transmission of the gent (TABLE 10).

The observations obtained for the $ACEF_2$ hybrids are difficult to interpret ccording to any genetic theory. Whereas more than 80 per cent of the reeding females of the A and CE strains had mammary cancer, the incidence ecreased from 91 per cent in the $ACEF_1$ mice to only 65 per cent for breeders f the $ACEF_2$ generation, and the respective percentage of cancerous nonreeders on the two generations were 37 and 17. If we assume that the mice f the A and CE strains are susceptible for mammary cancer, because of the igh incidences in breeders, the incidence in the breeders of the $ACEF_2$ generation would indicate a segregation of genes, suggesting that their susceptibility may not be produced by identical genes.

To secure additional information concerning the genetic constitution of CE mice for the development of cancer, another litter was fostered by a female of the cancerous Z(C3H) stock, and these CE females and their progeny were mated with males of either the I or JK stocks. The CE females with the agent showed a high incidence (TABLE 11).

Approximately 200 hybrids were observed in the various groups as either breeders or virgins. No significant difference was seen between the F_1 hybrids sired by males of the two sublines of the resistant I stock, and nearly 90 per cent of the CEz♀ × I♂ F_1 nonbreeders had mammary cancer at an earlier average age than was observed for breeders of the cancerous CE stock. Lower incidences were found among the hybrids of the CEz♀ × JK♂ cross. Data for certain other pertinent crosses are given for comparison.

Other data were secured by mating CEz females with males of the C57 black stock, subline 1, to obtain the so-called CEzB_1F_1 hybrids, of which some, including litter-mate controls, were continued as either breeders or virgins. Only preliminary data may be given for this cross, as 30 separated as virgins and 20 as breeders are living, and are at least 1 year of age. One nonbreeder has died with mammary cancer when 434 days of age and 33 CEzB_1F_1 breeders have had tumors, at an average age of 348 days; 5 died noncancerous. Thus, 57 per cent of all breeders have died with mammary tumors and 34 per cent are under observation. These preliminary data would indicate, in this group of hybrids, that the MTA and susceptibility for cancer were contributed by the CEz females, but that the hormonal pattern of the CE stock was not similar to the inducing hormonal pattern.

TABLE 12*

MAMMARY CANCER IN INBRED MICE AND THEIR HYBRIDS

Stock or generation	Cross	Virgin females				Breeding females			
		No.	Cancer (per cent)	Av. age in days		No.	Cancer (per cent)	Av. age in days	
				Cancer	Non-cancer			Cancer	Non-cancer
ANHF_1	A♀ × NH♂	128	16	567	689	68	43	428	617
ANHF_1-AxBC	F_1♀ × Ax♂	50	14	499	670	40	43	335	533
ANHF_1-ZbBC	F_1♀ × Zb♂	69	61	508	726	44	82	389	703
ZNHF_1	Z♀ × NH♂	62	94	495	639	48	90	345	490
ZNHF_1-AxBC	F_1♀ × Ax♂	38	30	575	688	30	83	352	652
ZNHF_1-ZbBC	F_1♀ × Zb♂	31	68	425	663	19	79	261	427
A	A♀ × A♂	111	4	450	573	569	84	330	387
Z(C3H)	Z♀ × Z♂	153	66	404	499	858	95	278	322
NH	NH♀ × NH♂	0				84	27	387	489

* Reproduced by permission from *Cancer Research.*[47]

Mice of the NH stock have been tested because it was determined that old intact NH females would develop adrenal cortical tumors,[78, 101] which could be accelerated by ovariectomy.[78, 79, 101]

The first cross to be made was between females of the A stock and NH males; although previous experiments had shown the transmission of the inherited susceptibility for mammary cancer as a dominant by mice of the inbred A strain,[27-29, 36, 45, 60] (TABLE 9) only 43 per cent of the ANHF breeders developed mammary cancer, and the incidence was not increased in the backcross hybrids to the A stock (TABLE 12). With the introduction of the inducing hormonal pattern by mice of the Z stock, higher incidences were found not only in breeders but also in the hybrids maintained as virgins secured by mating the ANHF_1 females with males of that strain.[47, 51] The NH stock exerted some influence in the average cancer ages.

To determine if mice of the NH strain might be susceptible for mammary cancer, NH animals were fostered by females of the cancerous Z(C3H) stock and, when these NHz females (with the MTA of the Z stock) were observed as breeders, only 27 per cent died cancerous at an average age of 387 days

The incidences were similar for females of the fostered generation and the descendants of the succeeding 4 generations. When compared with the ncidences observed in breeding females of other inbred stocks when they had the MTA, these data would indicate that mice of the NH stock were relatively nonsusceptible for the development of mammary cancer (TABLE 12).

Observations obtained in the hybrids derived by mating these NHz females with males of other stocks, including those of the F_2 and backcross generations, are tabulated in TABLE 13. In some, the numbers are too small to have significance. Certain groups may be compared with the reciprocal hybrids recorded in TABLE 12, the majority of which were observed during an earlier period.

TABLE 13

MAMMARY CANCER IN NHz FEMALES AND THEIR HYBRIDS

Stock	Observed as breeders			Observed as virgins		
	No.	Cancer	Cancer age	No.	Cancer	Cancer age
NHz	84	27%	387	0		
Iz or Ia	53	0%	—	0		
NHz♀ × I♂F_1	38	76%	319	12	16%	590
NHzIF_2	35	57%	273	0		
NHzIF_1♀ × I♂	31	61%	283	16	13%	421
NHzIF_1♀ × NH♂	25	52%	359	10	0%	—
NHz♀ × Zb♂F_1	85	93%	355	19	58%	461
NHz♀ × Ax♂F_1	54	69%	422	46	20%	490

If the NHz females were mated with males of the I stock, where the respective incidences were 27 and 0 per cent in breeding females with the MTA, the resulting NHzIF_1 hybrids had a high incidence as breeders, but a low incidence as virgins (TABLE 13). A reduced incidence (57 per cent) was observed in the breeders of the F_2 generation, yet the incidence was higher than found in either parental strain. A small number of backcross hybrids were obtained by mating the NHzIF_1 females with males of the parental NH or I stocks. Approximately the same incidence of tumors was found in the breeders of the two groups, but of special interest was the early cancer age seen in the backcross hybrids sired by males of the resistant I stock.

These data may not be interpreted at this time, and only suggestive explanations may be made. It may be that the hormonal pattern transmitted by mice of the NH stock had an inhibitory effect upon the development of mammary cancer, even in animals that are as susceptible as the ANHF_1 hybrids[47] and have the hormonal stimulation resulting from breeding and lactation. The relatively high incidence to be found in the $NHzIF_1$ breeding females could be explained on the basis that the NH mice were susceptible for mammary cancer, but that the "inhibitory" hormonal mechanism "masked" this inherited tendency for the disease and reduced the incidence (as was seen also in the $ANHF_1$ mice). The inducing hormonal pattern introduced by the nonsusceptible I males was dominant over the inhibitory hormonal mechanism of the NH stock, allowing the susceptibility of the NH mice to become evident and giving a high incidence of tumors.[50, 51, 55] If this might be the answer, the activity of the inherited susceptibility of the NH animals was influenced, even in breeders possessing the MTA, by another inherited factor controlling the hormones.

The effects of adrenalectomy and ovariectomy upon the development of mammary tumor in mice were demonstrated in the experiment of Shimkin and Wyman.[136] Attempts have been made to determine the basis of the inherited hormonal mechanisms by the transplantation of either ovaries or adrenals into either ovariectomized or adrenalectomized-ovariectomized animals. By transplanting tissues from mice of inbred strains into their F_1 hybrids, the grafts would be subjected to comparable pituitary stimulation and the hormones produced by the grafts would act upon and be metabolized by tissues having the same genetic constitutions. If significant differences were obtained, especially in the development of mammary cancer in the hybrids bearing grafts from the two parental strains, it might be assumed that the observations could be due to inherited hormonal effects associated with the grafts.

By the transplantation of ovaries into gonadectomized animals[60, 94, 95] the development of mammary cancer in the various groups indicated a difference in the inherent hormonal function of the ovaries from the two inbred strains. A variation in pituitary activity of the two strains was probably responsible for the morphological appearance of the ovaries, but after three months in the F_1 hosts the histology of the grafts was similar. By transplanting adrenals into ovariectomized-adrenalectomized mice it was found that the postcastrational adrenal alterations that developed in different inbred strains of mice were probably due to the inherent responsiveness of the adrenal tissue itself.[96]

In addition to postcastrational adrenal cortical lesions, it has been determined that gonadectomized animals of some strains may have enlarged pituitaries with basophile adenomas.[68, 140, 143] It has been suggested that the hormones supplied by the abnormal adrenals and pituitaries would produce, in susceptible animals possessing the MTA, mammary cancer not only in ovariectomized females but also in castrated males. Only a limited amount of data has been presented to support such a theory.

It has yet to be demonstrated that gonadectomized mice of any inbred strain or hybrid generation would develop mammary cancer when the virgin females of such a group had a low incidence, or did not have the mammary cancer-inducing inherited hormonal pattern. As may be seen from the incidences of mammary tumors given in the various tables, it has become evident "that differences in physiological hormonal effects of the various postcastrational changes may be of greater importance in the etiology of mammary cancer than the anatomical appearance of the adrenal and/or pituitary lesions and, since different inherited hormonal patterns have been demonstrated in various strains of mice, further experimental studies are needed to determine the physiological relationship of these to the development of mammary cancer in breeding and virgin females and gonadectomized animals."[49]

Summary

Data have been examined concerning genetic concepts in the etiology of spontaneous mammary cancer in mice bearing upon the following aspects of the disease.

The inherited susceptibility for the development of spontaneous mammary cancer has been found to be transmitted equally well by both males and females of cancerous stocks. In one cross, linkage was demonstrated between the gene-producing brown coat color and one of the susceptibility genes. While the incidence may be reduced with the elimination of the MTA, this does not alter the inherited susceptibility, and the incidence may be restored by the administration of the agent.

The genetic make-up of animals of an inbred strain for the genesis of spontaneous mammary cancer may be determined *only* by testing mice that possess the MTA and have been subjected to hormonal stimulation adequate for the induction of these tumors. It has not been ascertained if the inherited susceptibility for the genesis of the usual type of spontaneous mammary cancer may be similar to that required for the genesis of mammary tumors in agent-free animals, including mammary tumors induced by carcinogenic agents.

Several inherited hormonal patterns have been described; one of these plays a role in the induction of mammary tumors in virgin females, while another may inhibit their development in breeding females of susceptible stocks with the agent. Preliminary data indicate that the inducing pattern may be dominant over the inhibitory mechanism, although the latter may exert some influence over the average cancer age. Some of the physiological effects of these hormonal patterns have been revealed by the transplantation of endocrine organs into suitable test animals, but further studies are indicated.

Observations obtained in several hybrid crosses suggest that the same genes do not control the inherited susceptibility for mammary cancer and the mammary cancer-inducing inherited hormonal influence, for the inducing hormonal pattern may be transmitted by mice that are resistant to the development of mammary cancer. In addition, mice that are susceptible for the disease may transmit the inhibitory hormonal influence, and this tends to mask the inherited susceptibility.

Data have been obtained showing that the incidence of spontaneous mammary cancer may be no higher in breeding susceptible females having the MTA than has been observed in agent-free susceptible breeders, from which the agent cannot be recovered by biological assay.

Following the introduction of the MTA into agent-free females by such methods as nursing, injection, and male transmission, the progeny of such females will have an increased incidence and acceleration in the time of appearance of the tumors, and the agent can be demonstrated to be present in the tumors from the infected mothers and their offspring.

Susceptible females may not show similar incidences and average cancer ages after they have been nursed by females of different cancerous stocks. The agent from one cancerous stock may have different antigenic properties after being introduced into mice having another genetic constitution, and antiserum produced against the latter may not neutralize the MTA obtained from the original strain. The activity and antigenic characteristics of the agent may become altered when propagated for many passages in a transplanted mammary tumor.

With possible different agents, or with agents having different tumor-inducing activities, it has become increasingly difficult to interpret some experimental data, especially to correlate the observations obtained in different experiments. It is evident that comparable factors transmitted by two inbred strains need not be determined by the same genic make-up, although common genes may exist.

Further information must be obtained about the transmission and propagation of the MTA since it has been demonstrated that mice of different sublines of a resistant stock, or even young born in the same litter, may not transfer the agent from a cancerous stock with the same effectiveness and/or activity, and the data may be quite different after females of another cancerous stock are used as the fostering mothers. Also, females of one line may transfer the agent for many generations, while the MTA cannot be demonstrated in mice of another line of the same stock after a few generations.

It is not known whether the sensitivity of agent-free females of various strains to infection with the agent from males of cancerous strains may be influenced by another genetic complex. The problem must be approached with the knowledge that the ability of males of cancerous strains to infect agent-free females may become altered by foster nursing, or where the males obtained the MTA of the fostering cancerous stock.

The isolation of strains into sublines should provide new animal material for many types of investigation, but each study should have adequate controls, and mice of one line should not be considered as suitable controls for a study where mice of another line are employed. Animals of sublines known to be different genetically should never be tabulated together to give larger numbers for statistical purposes.

The MTA may "appear" in mice of either susceptible or resistant stocks not nursed by females possessing the agent, injected with extracts, or mated with males of cancerous stocks, and the agent may be transferred and propagated for generations. Should this occur in susceptible mice a high cancerous stock will result.

Further studies should be concerned with the possible genetic control o other aspects of the genesis of mammary cancer, including the sensitivity o various tissues to hormonal stimulation, the development of different type of mammary tumors, and the possibility that the MTA may be of genic origin and operate in the individual through enzymelike control of hormonal metabolism and/or production or through other processes.

There are no data to suggest that the observations made upon mice may apply to breast cancer in humans, especially concerning a milk-borne infectious agent as one of the etiological factors. Should one be present and have the characteristics of the mammary tumor agent of mice, it would be difficult to demonstrate its presence, for it could be transferred at coitus, in the blood, perhaps by saliva, and also by nursing, and it would be active only in the development of certain types of breast cancer. As in mice, it could be propagated and transferred for several generations in resistant individuals without causing cancer, and the incidence could be as high in certain groups lacking the agent as in others where it was present.

We should not, however, lose sight of the fact that the ultimate purpose of cancer research is the solution of the problem in humans for, unless man were subject to the disease called cancer, there would be little need or opportunity for cancer research.

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SOME ASPECTS OF DRUG RESISTANCE IN NEOPLASMS

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For some time drug resistance has been recognized as a very serious limitation to the continued usefulness of compounds in certain bacterial and parasitic infections. From definitive evidence obtained at the experimental level it is now clear that the problem of resistance in neoplastic cells is a perpetual threat to the successful use of therapeutic agents. By inference, the eventual failure of drugs initially effective, particularly in childhood leukemias, may be attributed to the emergence of drug-resistant populations of neoplastic cells.

The development of drug resistance in neoplastic cells is a far greater hazard than that encountered in bacterial populations for these reasons:

(1) Defense mechanisms of the body are of little or no importance in neoplastic disease. Thus, effective chemotherapy requires destruction (or inactivation) of most, or all, neoplastic cells.

TABLE 1

VARIATION IN RESPONSE OF ASCITIC LEUKEMIAS TO VARIOUS DRUGS

Neoplasm	Morphologic form	Strain	Response to				
			Antifolics	Antipurines*	Glutamine† antagonists	Pyrimidine‡ antagonists	Cortisone
L-1210	Lymphocytic	DBA/2	++	+	−	±	−
L-4946	Lymphocytic	AKR	++	−	−	±	−
70429	Plasma cell	C3H	+	−	++	++	−
H-5530	Acute granulocytic	C58	−	−	−	++	−
P-288	Lymphocytic	DBA/2	++	±	±	+	−
P-1081	Chronic granulocytic	DBA/2	−	−	±	++	−
P-1798	Lympho-sarcoma	BALB/c	+	++	±	+	++
P-329	Histiocytic	DBA/2	±	±	−	++	−
P-815	Mast cell	DBA/2	−	−	++	++	−

Symbols: − = little or no inhibition; ± = approximately 50 per cent increase in survival time; + = 100 per cent increase; ++ = 200 per cent or more increase.

* Thioguanine, 6-mercaptopurine, and 8-azaguanine.

† Azaserine and DON.

‡ 5-Fluorouracil and 5-fluorouridine.

(2) Means of combating resistant populations in bacterial infections may be expected to emerge from the rapid discovery of new antibiotics with different mechanisms of action, from the possibility of using massive doses of drugs continuously, and from the employment of association of drugs. Very few drugs are known to be effective for neoplastic diseases, and the serious limitations imposed by normal cells of the host usually prevent attainment of maximum effective concentrations in the body.

(3) The interference of certain antibiotics in metabolic sequences of bacteria, but not of mammalian cells,[1] allows a selective toxicity probably never to be encountered in neoplastic cells.

These facts, along with indications that resistance in certain neoplastic populations is mutational,[2] appear to have fatalistic implications. However, as information is gained relating to the origin and mechanisms concerned with resistance in both bacteria and neoplasms, it would appear that means will be found of precluding, of circumventing, or of exploiting the phenomenon. Certain explicit examples will be cited later.

TABLE 2

RESISTANCE IN NEOPLASMS DEVELOPED THROUGH THE USE OF SEVERAL CARCINOSTATIC AGENTS*

Neoplasm	Designation	Species and strain	Compound used	Resistance (R) or dependence (D)
Lymphocytic leukemia	AK-4	AK mice	A-Methopterin	R
Lymphocytic leukemia	I	C58 mice	A-Methopterin	R
Lymphocytic leukemia	L-1210	DBA/2 mice	Various 4-amino PGA antagonists	R and D (4 lines)
Lymphocytic leukemia	L-1210	DBA/2 mice	8-Azaguanine	R and D
Lymphocytic leukemia	L-1210	DBA/2 mice	6-Mercaptopurine	R
Lymphocytic leukemia	L-1210	DBA/2 mice	Thioguanine	R or D
Lymphocytic leukemia	L-1210	DBA/2 mice	Thioguanine and A-Methopterin	R to both
Lymphocytic leukemia	L-5178	DBA/2 mice	6-Mercaptopurine	R
Lymphocytic leukemia	L-5178	DBA/2 mice	A-Methopterin	R
Lymphocytic leukemia	L-4946	AKR mice	A-Methopterin	R
Lymphocytic leukemia	P-288	DBA/2 mice	A-Methopterin	R
Lymphocytic leukemia	P-388	DBA/2 mice	A-Methopterin	R
Lymphosarcoma	P-1798	BALB/c mice	Cortisone	R
Plasma cell leukemia	70429	C3H mice	Azaserine, DON, A-Methopterin, and 6-azauracil	R and D (5 lines)
Mast cell neoplasm	P-815	DBA/2 mice	DON	R
Fibrosarcoma	Sarcoma 180	Swiss mice	6-Mercaptopurine	R
Carcinoma	Ehrlich carcinoma (ascites)	Swiss mice	N-methyl colchicamide	R
		Swiss mice	N-methyl formamide	
Fibrosarcoma	Walker rat sarcoma	Rat	N-acetyl derivative of N mustard	R
Carcinosarcoma	Walker	Wistar rat	TEM	R (2 lines)
Fibrosarcoma	Yoshida sarcoma (ascites)	Rat	Methyl-bis(β-chloroethyl)-amine-N-oxide	R
Carcinoma	755	C57Bl mice	6-Mercaptopurine	R
Carcinoma	TA-3	CAF_1 mice	Azaserine	R

* Many lines of transplantable lymphomas are in existence that show a natural resistance to the many agents discussed here. In fact, two lymphocytic neoplasms, L-3054 (C58 mice) and P-433 (DBA/2 mice) apparently grow optimally in the presence of 8-azaguanine and 6-mercaptopurine, respectively.

The experimental development of lines of neoplastic cells showing resistance to and, on rare occasions, dependence (partial) upon certain carcinostatic drugs is now established.[3] Most emphasis has been placed upon the leukemias and lymphomas because of the ease of handling them and their striking responses to several classes of antimetabolites (TABLE 1). It may be seen from TABLE 2, however, that resistance to a variety of agents has been achieved, experimentally, in several different morphologic forms of neoplasms, in solid as well as ascitic forms.

Development of Resistant Lines

In general, the failure of neoplastic cells to respond to a drug after a striking initial sensitivity may be the result of variables either in the host or the cells. Burchenal *et al.*[4] first described the phenomenon of resistance to the folic analogue, A-Methopterin, in a population of cells of an acute lymphocytic leukemia in the mouse. In the same year we were able to select resistant and dependent leukemic cells with various 4-amino substituted folic analogues,[5] and subsequently with several purine analogues, using the common morphologic form of lymphoma of the mouse, lymphocytic leukemia.[6-9] Our continuing efforts are directed also to other forms of lymphomas available as ascitic neoplasms, such as type A and B reticular neoplasms (monocytic and histiocytic leukemias, reticulum cell sarcoma), plasma cell neoplasms (including multiple myelomalike forms), and granulocytic leukemias, which respond quite specifically to antileukemic agents.

TABLE 3

DIFFERENCES OBSERVED IN TWO LINES OF THE LYMPHOCYTIC NEOPLASM L-1210

	Sensitive (S)	8-Azaguanine-dependent (8-AG-D)
Ascitic fluid	Bloody	Nonbloody
Mean survival following inoculation of 1 × 10^6 ascitic cells	7.7 days	12.7 days
PGA metabolism		
CF content of cells	0.52 μg./gm. cells	2.87 μg./gm. cells
PGA to CF conversion	23.5 μg./gm. cells	146.5 μg./gm. cells
A-Meth. inhibition of PGA to CF conversion*	x	x × 7
Incorporation		
Na C^{14} formate (CNA)†	47 μc./mole C	97 μc./mole C
2,6-Diaminopurine-2-C^{14} (CNA)	91 μc./mole C	12 μc./mole C
8-Azaguanine-2-C^{14} (RNA)‡	2.86 μc./mole C	314 μc./mole C
Drug response		
A-Methopterin	+	++++
8-Azaguanine	+	–
Azaserine	±	++

Symbols: — = no increase in survival time; each + = 100 per cent increase over controls.

* Unpublished data of Nichol and Law (see also Nichol[26]).

† CNA = Combined nucleic acids.

‡ See Skipper.[52]

In our laboratory we have developed populations of cells resistant to such other compounds as azaserine, azanorleucine (DON), *N*-methylformamide, 6-azauracil, and cortisone.[10, 11]

One specific transplantable lymphocytic neoplasm in DBA/2 mice, L-1210, has received considerable attention in our laboratory. This leukemia has now been carried through 400 consecutive serial transfers, killing regularly in 7 to 8 days and exhibiting logarithmic growth as determined in the ascitic form. This neoplasm is quite sensitive to antifolic and antipurine drugs, several antibiotic beers, glutamine antagonists, and other antimetabolites. It is of interest to note that during this long period of transfer no detectable changes in response to drugs have been observed, indicating that the endogenous environmental variables within the host are relatively nonselective as regards drug sensitivity. In contrast, selective pressures applied to the population of sensitive cells through the use of certain antimetabolites have resulted in the development of variant sublines differing from the control sensitive line in various physiological and biochemical aspects, as well as in response to the specific selecting drug (TABLE 3). Several sublines resistant to, or dependent upon, the drug used for selection have been described.[5-9] Three sublines of L-1210, notably the AM-D (A-Methopterin-dependence), 8AG-D (8-azaguanine-dependence), and TH-R (thioguanine-resistance) have been carried 30, 55, and 50 transfers, respectively, in the absence of the drug,

TABLE 4

RESPONSE OF SENSITIVE (S) AND THIOGUANINE-RESISTANT (TH-R) SUBLINES OF LEUKEMIA L-1210 TO VARIOUS COMPOUNDS

L-1210/S

	Number of experiments	Total mice	Dosage, mg./kg.		Mean survival, days	Per cent increase survival
			Individual	Total		
Controls	14	105	None		7.7 ± 0.08	—
Thioguanine	13	98	7.5–10	30–40	16.3 ± 0.56	112
6-Mercaptopurine	13	106	75	400–600	14.8	92
8-Azaguanine	8	72	75	600	12.3	
Purine	2	16	75	400–600	10.6	
Chloropurine	2	16	75	400–600	10.6	
A-Methopterin	5	41	3	24	17.2 ± .47	124
			L-1210/TH-R*			
Controls	8	64	None		8.5 ± 0.14	—
Thioguanine	8	65	7.5–10	30–40	8.3 ± 0.10	—
6-Mercaptopurine	3	24	75	600	8.7	—
8-Azaguanine	3	24	75	600	8.7	—
Purine	4	32	75	400–600	9.2	—
Chloropurine	4	33	75	400–600	9.1	—
A-Methopterin	4	32	3	24	39.2 ± 7.5	361

The L-1210/TH-R subline was developed by consecutive serial transfer in DBA/2 mice receiving increasing levels of thioguanine. Seven consecutive transfers, beginning with the 246th transfer of L-1210/S were required. TH/R line was carried without drug.

thus showing these characters to be stable, irreversible, and heritable.[8] In contrast, resistance to A-Methopterin in a plasma cell neoplasm, 70429, in certain cases and under certain conditions has failed to behave as a stable character.[12] This will be discussed later.

The response of a derived line of the leukemia L-1210, L-1210/TH-R (resistant to thioguanine) is shown in TABLE 4. Here resistance is complete; optimal growth occurs in the presence or absence of the drug. In other neoplasms, however, different levels of resistance are observed, as may be determined by many different parameters of growth, such as local lymphomatous growth, infiltration into peripheral blood and hematopoietic organs, and total number of ascitic neoplastic cells.

The character of partial dependence, wherein populations of derived cells appear to grow optimally in the presence of a drug, has been observed in only a few cases.[13] Host variables may intervene in some situations, giving the impression of dependence, but a true partial dependence has been observed in neoplasm L-1210 to 8-azaguanine,[13] and in the plasma cell neoplasm 70429 to A-Methopterin.[12] In the latter case, lysis of the neoplastic cells is seen to occur in the absence of drug. Heritability of the character here is not yet firmly established.

Origin of Resistant Variants

The development of antineoplastic agents has brought to the foreground new aspects in the evolution of neoplastic cells. Intercellular variability of neoplastic cell populations gains support from many facts: the attainment of autonomy, loss of histocompatibility genes, immunoselection in neoplasms, ascites transformation, fluctuations in such factors as cell size and chromosome numbers, increase or decrease in specificity, and changes in behavior in neoplasms through microisolation of clonal sublines. Thus, recognition of the heterogeneity of neoplastic cell populations and an understanding of the role of applied and natural selection as they relate to the above are major problems confronting the oncologist.

Sufficient evidence is now at hand from studies of neoplastic cell populations in the experimental animal to substantiate the statement that drug failure results from changes within the cells. The possibility of the influence of host variables must still be considered in certain cases, and this potentiality probably has not received adequate attention. Hepatic detoxication, the development of a more efficient renal excretory mechanism, and the influence of such factors as degradative enzymes and viruses conceivably could account for drug failure.

In the laboratory, as in the clinic, it has been observed that within a given morphologic form of neoplasm (for example, childhood leukemia and lymphocytic leukemia of the mouse), nearly 50 per cent of the individual neoplasms respond in some degree to folic analogues; others are naturally resistant to the maximum attainable concentration of drug. This situation is equally true for other drugs (TABLE 1). The selective action of the drug on leukemic cells, in contrast to normal cells, varies considerably from individual to individual, and the percentage of the population of leukemic cells susceptible to increasing concentrations of a drug probably follows a normal distribution curve. If the barrier to a higher dosage of drug imposed by susceptible normal cells occurs at the average sensitivity of the leukemic population, no beneficial effects of the drug may be seen. However, if the barrier (maximum concentration attainable) occurs at a concentration permitting only a few cells to survive, a good response or remission results. The growth of the remaining cells would eventually give rise to a resistant population.

Two general alternative possibilities must be considered in the response of a population of sensitive neoplastic cells to a drug (these need not be mutually exclusive):

(1) *Physiological adaptation.* The drug induces a change in the population of cells, and adaptation of an extranuclear nature results. Where drug resistance is specifically related to enzyme function, it is possible that either an "induced enzyme synthesis" or an inducer-inactivating enzyme[15] may be involved.[14] The questions of adaptive significance and of permanency of the change must be raised, however. Other mechanisms implicating physiological adaptation have been reported.[16] An interesting example of cellular adaptation related to the Barrett-Deringer phenomenon, a unique case of "tumor progression," has been reported recently by the Kleins.[51]

(2) *Genetic adaptation.* Individual cells of a given genotype are unable to cope with the new environment. Mutation thus provides a basis for new heritable variation. If these mutants are able to propagate themselves, a new population arises from clones better fitted to survive. Mutants arising independently of the drug are selected and isolated, and a new population results.

The roles of mutation and selection in drug resistance in bacteria have been sufficiently documented both by direct and indirect methods to point convincingly to the fact that an important mechanism in most resistant forms studied to date is one of genetic adaptation. Direct evidence obtained by Demerec,[17] Newcombe and Nyholm,[18] and Lederberg[19] through genetic exchanges implicates specific genes in resistance of *Escherichia coli* to streptomycin. The drug-independent origin of mutants resistant to phage, streptomycin, and isoniazid have been reported. More recently, Hotchkiss[20] has shown that purified preparations of DNA derived from resistant mutants can transmit the property of resistance to penicillin or streptomycin in pneumococci.

A highly selective mutagenic action causing a cell, bacterial or neoplastic, to change only its resistance to an inducing drug is highly unlikely for many reasons, and recent reports by Akiba[21] and Szybalski[22] of a streptomycin-induced purposeful change appear now to be discredited.[23]

An indirect method introduced by Luria and Delbrück,[24] the "fluctuation test," which distinguishes between physiological and genetic adaptation in bacterial populations, has been adapted to a study of the origin of resistance to A-Methopterin in the L-1210 lymphocytic neoplasm.[2] It was concluded from this study, in which, with other collateral evidence, resistant sublines appeared in the absence of the drug, that mutation and selection constituted the mechanism by which resistance arose. Increase in resistance was seen to occur in a stepwise fashion, and the character was maintained in the absence of the drug.

Patterns of Resistance

Resistant and dependent variants obtained through the use of other antimetabolites such as Aminopterin, 6-mercaptopurine, thioguanine, and 6-azauracil appear also to arise in a discrete, stepwise fashion, resembling the penicillin pattern[25] of resistance.

Most neoplastic cell populations in our laboratory have shown some degree of adaptation to the available therapeutic drugs. The degree of resistance attained by neoplastic cells in man or in the experimental animal appears to be relatively much lower than in bacterial populations, probably because the intensity with which these drugs can be used is seriously limited. In two instances the levels of resistance attained are in the order of tenfold increases.[10] As described by Nichol,[26] from the standpoint of practical chemotherapy an emergent population of resistant cells requires only a degree of sensitivity to the drug analogous to that of certain normal tissues; slight changes in the characteristics of neoplastic cells may be sufficient to confer resistance.

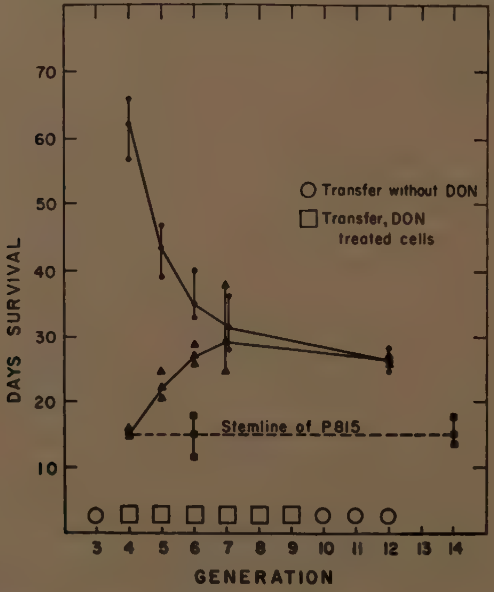

Figure 1. History of the development of resistance to DON (azanorleucine) in a mast cell neoplasm P-815.[27]

The emergence of a sudden adaptation similar to streptomycin or isoniazid resistance in microorganisms (single-step pattern) has been observed in our laboratory in a plasma cell neoplasm (designated 70429) in C3H mice.[10] Recently Potter[27] has observed a mast cell neoplasm (P-815) to develop resistance to DON in a similar pattern. That the response to DON may be a facultative rather than an obligatory single-step pattern is seen in FIGURES 1 and 2. Here, at an earlier transfer generation, the evolution of a resistant population of the mast cell neoplasm P-815 was seen to occur in several steps. Resistance in the neoplasm 70429 to high levels of the glutamine antagonists, azaserine and azanorleucine, have been attained during

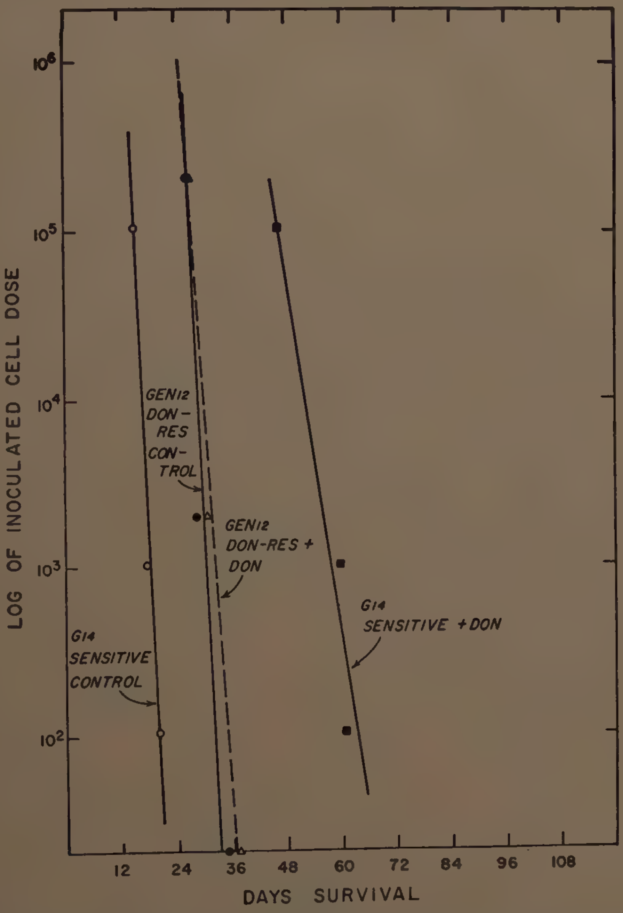

FIGURE 2. Response of DON-resistant neoplasm P-815 to daily treatment with DON, in comparison with the stemline. Different cell inocula were used here. See FIGURE 1 for the evolution of the resistant line.

the course of a single transfer generation. TABLE 5 reveals the changes that occur in the response of this chronic neoplasm during selection with azaserine. Resistant populations of cells also were isolated from sites of dissemination in the animal from such areas as subcutaneous connective tissue and cervical lymph nodes. In certain instances these populations of cells exhibited a permanent and heritable resistance to the glutamine antagonists; other popu-

TABLE 5

AZASERINE RESISTANCE IN THE PLASMA-CELL NEOPLASM 70429*

Generation	Cell dose × 10⁶ inoculated	Number of transfers without drug	Treatment, mg./kg. × days		Average day of death		
			Azaserine	DON	Control	Azaserine	DON
12	1.72	Sensitive	2.5 × 53	—	29.1	65.1	—
13 20 46	2.3 1.05 1.17	— 6 32	2.5 × 14 5 × 25 5 × 23	— .125 × 16 —	25.6 41.0 22.2	17.7 31.5 25.0	— 29.2 —
43	1.1	Sensitive	5 × 70	—	28.5	74.6	—

* Resistance to azaserine developed through passage of sensitive 70429 in mice receiving azaserine. Resistance developed in one transfer, the twelfth, and persisted through transfers in nontreated mice.[10]

lations isolated in this manner were still sensitive to these drugs.[10] FIGURE 3 shows a comparison of these two types of patterns of response in neoplastic cells developing resistance to therapeutic drugs.

Instability of resistant populations in certain experimental situations, particularly of the plasma cell neoplasm 70429, have been encountered in our laboratory. Incomplete selection appears to be an explanation in some cases; in other cases, after severe selective pressures with folic analogues through several transfer generations, resistant populations are seen to revert

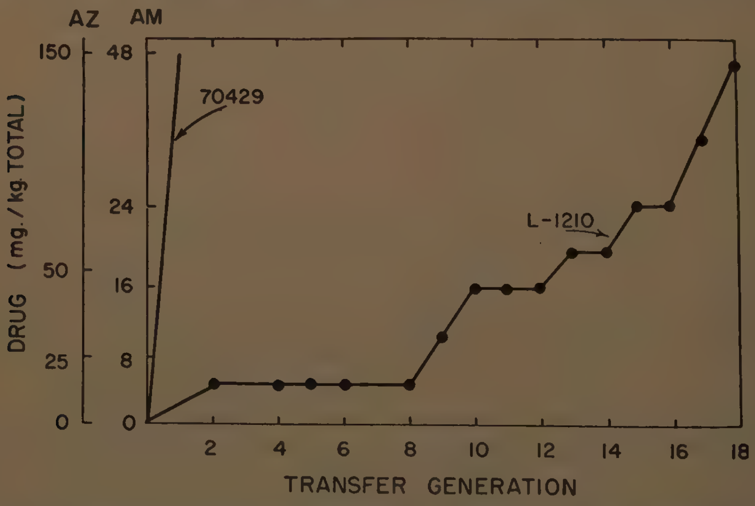

FIGURE 3. Patterns of resistance developed in two neoplasms of mice following selection with antileukemic agents. Plasma cell neoplasm 70429 was treated with azaserine (AZ); lymphocytic leukemia L-1210 was treated with A-Methopterin (AM).

lowly to sensitivity.[27] At the moment the difficulties in isolating individual omponents of a population of neoplastic cells and studying their relationhips one to the other are obstacles encountered in attempting to explain ome of the phenomena observed.

By sexual recombination studies in *E. coli* K-12, Lederberg[19] has shown hat streptomycin resistance behaves as a recessive character. It is of nterest to note that, in our laboratory, development of resistance to A-Methopterin in a lymphocytic leukemia (designated P-288) was developed in a stepwise manner in several transfer generations and has maintained its resistance character, in the absence of the drug, for more than 20 transfers.[11] This neoplasm, in contrast to most we have used, is tetraploid. If the character in this instance is presumed to be of genetic origin, it must be dominant or semidominant.

It is impossible at the moment to define in genetic terms the precise mechanisms concerned in the development of insensitivity to drugs. Neoplastic cells are somatic cells and, as such, are generally thought not to lend themselves to genetic analysis. We must consider the possibility of the development of procedures analogous to sexual processes in certain bacteria, or of the occurrence of genetic variants brought about by agencies similar to the transforming and transducing factors known to play a role in the evolution of bacterial types. The recent development of specific markers in neoplastic cells and the progressive growth of clones of neoplastic cells in tissue culture are encouraging in this respect. It should be pointed out that a system does exist by which one may distinguish cellular (somatic) variants that differ from the original type with respect to allelic substitution at a single gene locus, the histocompatibility locus.[28]

The possibility that physiological adaptation plays a part in the development of nonsensitivity in neoplastic cells to drugs must be considered. Mention was made that the plasma cell neoplasm 70429 became resistant to the folic analogue A-Methopterin. In the absence of the drug, unlike other variant lines observed, reversion to sensitivity occurred in some populations even after strong selective pressures had been exerted. The mechanisms underlying this form of adaptation as distinct from the stable, irreversible, heritable types are undoubtedly quite dissimilar. Therefore, at the experimental level, when nonsensitivity to a drug arises, it should be determined to what extent the population of neoplastic cells has responded to the adaptive stimulus and, if the response is discontinuous, to what extent it is due to a heritable differentiation among the cells of that population with respect to ability to adapt. Successful therapy depends to a great extent upon this knowledge. These distinctions can be made in experimental neoplasms. The recent success attained in tissue culture of neoplasms (particularly lymphomas) and heterologous transfer of human neoplasms would make it appear technically feasible to do so in man.

Attempts at Circumventing or Exploiting Resistance

Spontaneous mutations can neither be prevented effectively nor reversed in a directive manner, although recent reports on the reduction of spontaneous mutations by certain nucleosides is hopeful.[29] Difficulties would be expected in any attempt to interfere with the origin of resistance dependent upon genetic change. The origin of resistance through a mechanism of physiological adaptation would in all probability be easier to control.

A more likely time to attempt the control or elimination of resistant neoplastic cell populations is after they have made their appearance.

(1) The use of combinations of drugs is based on the hypothesis that resistance to one drug may be eliminated by use of another. The opportunity for the occurrence of two or more mutations conferring resistance to each drug is thus severely limited. Recent *in vitro* evidence obtained by Szybalski and Bryson[30] concerning the frequency of double mutants of *B. megaterium* following exposure to isoniazid and PAS is pertinent here. Others[31, 32] have made use of a different rationale in their approach to combination-drug therapy.

The spontaneous appearance of doubly or multiply resistant bacterial mutants is indeed rare. Theoretically, the probability of a doubly resistant mutant arising is equal to the product of the individual mutation rates. In our hands doubly resistant neoplastic cells have been obtained, but only after the drugs (A-Methopterin and thioguanine) were given in sequence.[9]

Combined therapy has been unusually successful in certain neoplasms with certain combinations of drugs. An example of striking potentiation of action is shown, using a combination of A-Methopterin and the purine antagonist, 8-azaguanine, in inhibition of growth of the lymphocytic neoplasm L-1210 in DBA/2 mice.[33] Other examples of synergism in the partial control of neoplastic diseases have been published.[34-36] Of interest are examples of striking inhibition of neoplastic cell growth, particularly among some lymphomas, using antimetabolites, none of which is regularly inhibitory, particularly the combination azaserine and 6-mercaptopurine.[11] In this connection one group of investigators has pointed out the difficulties that may be encountered in attempting to assess potentiation of drugs, especially when given at near maximum tolerable levels.[37]

Combinations of antimetabolites are now receiving attention in the treatment of childhood leukemias. It should be appreciated that the success of such regimens will be determined only with adequate numbers of patients, since many are refractory to each of the compounds employed.

Interaction of drugs at the physiological level may range from synergism to additive effects, indifference, or even antagonism. On the basis of present knowledge concerning mechanisms of action of antineoplastic agents, it would appear premature to predict what combinations of drugs would effectively eliminate or effectively suppress resistant forms, even for a given morphologic form of neoplasm. Much more basic information is needed concerning cross-resistance, collateral sensitivities, and response to combinations of drugs in an array of different experimental neoplasms. The experimental situation necessary is one containing several variant sublines of neoplastic cells developed through selection. This is a difficult problem, and it entails a fairly large-scale screening of neoplasms in their sensitivities to drugs and the systematic development of resistant sublines. Inferences concerning such actors as origins and mechanisms of resistance and the use of associations of drugs may be gained in this manner.

(2) The use of combinations of drugs in sequence, or given simultaneously, s a question for debate. Theoretically, combinations of drugs should be given simultaneously if resistance to the specific drugs is known to have a genetic basis. In exceptional situations it is clear that drugs given in sequence may be advantageous. Collateral sensitivity to antifolic compounds is known to occur in populations of leukemic cells made resistant to such antipurines as 8-azaguanine, 6-mercaptopurine, and thioguanine.[8] A striking inhibition of leukemic cell growth is achieved when such resistant sublines are then treated with 4-amino substituted folic analogues (TABLE 6). Some evidence also is available to indicate that greater potentiation of effects is noted clearly in certain neoplasms given a course of treatment with 6-mercaptopurine followed by A-Methopterin before resistance develops.[11] These results appear to indicate the existence of an interrelationship between the metabolic pathways affected by the two types of antimetabolites. The changes in metabolism associated with collateral sensitivities and with increased effectiveness of the sequential use of drugs deserve more intensive study. The observations recorded to date indicate that the metabolism of neoplastic cells may indeed be manipulated in a manner that may amplify the meager basis for selective toxicity with which we are faced in attempting to control neoplastic diseases.

TABLE 6

INCREASED SENSITIVITY TO A-METHOPTERIN OF LEUKEMIC LINES TRANSFORMED THROUGH THE USE OF ANTIPURINES

Compound	L-1210/S		L-1210/8AG-D		L-1210/8AG-R		L-1210/6MR	
	Number	Survival	Number	Survival	Number	Survival	Number	Survival
None	145	8.0 ± 0.06	68	15.8 ± 0.45	42	10.2 ± 0.11	42	9.9 ± 0.10
8-Azaguanine	56	12.4 ± 0.20	74	12.1 ± 0.23	42	9.9 ± 0.09	—	—
6-Mercaptopurine	86	14.8 ± 0.18	—	—	—	—	42	10.1 ± 0.10
A-Methopterin	51	17.1 ± 0.69 (117 %)	81	63.1 ± 3.2* (300 %)	32	36.9 ± 1.5† (269 %)	34	27.4 ± 0.90 (175 %)

Dosage schedules: 8-azaguanine, 75 mg./kg. × 8 days subcutaneously; 6-mercaptopurine, 75 mg./kg. × 7 days subcutaneously; A-Methopterin, 3 mg./kg. × 8 days intraperitoneally. Treatment was begun 48 hours after inoculation of 8 × 10^5 leukemic cells intraperitoneally.

* Thirty nine mice (48.2 per cent) were negative at 120 days.

† Five mice (16 per cent) were negative at 120 days; survival time was determined at 90 days for all negative animals (see also TABLE 4).

Knowledge obtained to date through the use of the limited number of chemotherapeutic agents against experimental lymphomas indicates a wide degree of cross-resistance patterns. Neoplasms resistant to one antipurine, for example 6-mercaptopurine, are resistant to all antipurines and to the nucleosides available for testing; neoplasms resistant to one folic analogue are resistant to all folic analogues;[3] resistance to one glutamine antagonist (DON) is accompanied by cross-resistance to all other tested glutamine antagonists.[10] Similar observations have been reported in children in whom relapse has occurred with A-Methopterin.[38] It would appear from these observations that the number of potentially useful antileukemic agents may be appreciably limited by cross-resistance.

(3) Choice should be made of a drug that presents a multistep pattern of resistance, in preference to one giving rise to a single-step type. Theoretically at least, such drugs should be used at an effective concentration and continually. Decrease of the concentration below the effective level will permit accumulation of resistant first-step mutants and allow the occurrence of higher levels of resistance. Control of these neoplastic cells is therefore made more difficult. In view of the toxic effects of commonly used antileukemic compounds on rapidly regenerating epithelium of oral surfaces and intestine and on rapidly dividing cells of the bone marrow, it is questionable whether effective levels of such drugs are ever attained. Furthermore the difficulties in maintaining adequate levels of drugs commonly used are apparent.

Some attempts have been made recently to increase the levels at which drugs may be administered. Periodic replacement of damaged normal tissues such as bone marrow has been reported to be effective in increasing maximum tolerable levels with concomitant increase in life expectancy.[39] Success of such a program may be visualized for drugs such as thioguanine, which are toxic for specific tissues. Pathological changes are limited, for the most part, to the bone marrow following administration of this antipurine.[40] A selective protection for normal tissues in leukemic mice has been reported through the use of metabolites administered during the course of antimetabolite therapy—folinic acid in folic-antagonist treated mice and certain purine nucleosides and nucleotides in purine-antagonist treated mice.[41, 42]

It has been noted on several occasions by Burchenal,[43] and in our laboratory,[11] that experimental production of resistant populations in certain neoplasms to 6-azauracil and to DON* has been difficult to achieve. If these observations mean that the rate of mutation to resistance is exceedingly low in these experimental situations or that survival values of resistant forms are poor, the possibility appears real that certain drugs may be the most desirable in efforts to inhibit neoplastic growth.

Possible Mechanisms for Drug Resistance in Neoplastic Cells

The lack of a detailed understanding of the mode and site of action of drugs in common use in the treatment of neoplastic diseases has limited an approach to an early solution of the nature of resistance to any given drug. Several promising leads have been obtained, and model systems that provide for detailed study are available.

Studies in bacterial drug resistance implicate the following various mechanisms, some of which may be suggested as being operative in resistance in neoplasms, and others of which may be excluded: (1) altered accessibility to drug due to changes in permeability or transport mechanisms; (2) increased formation of a competing metabolite; (3) increase in the amount or efficiency of the enzyme using the metabolite; (4) decrease in the relative affinity of the susceptible enzyme for the drug (antimetabolite) as compared with the affinity for the metabolite; (5) development of alternative metabolic pathways; (6) inactivation of the drug; and (7) selection of heteroploid variants or other cytologically altered cells.

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* More recently, attempts to develop resistance in several neoplasms to 5-fluorouraci and 5-fluorouridine have been unsuccessful after many transfers in treated hosts.

Some information, particularly in bacterial systems, has come to light indicating in several instances that resistance results from inability of inhibitors to transfer across the cell membrane.[44, 45] In this connection it is interesting to note that Davidson[46] has found that the antipurines 8-azaguanine and 6-mercaptopurine enter L-1210/8-AG-R (8-azaguanine-resistant) cells freely. Nonsensitivity in these neoplastic cells would appear not to be related to reduced permeability.[46]

In only one instance in our laboratory is there a possibility of selection of heteroploid variants. Lampkin and Potter[12] observed an increased mean nuclear size in the cortisone-resistant population of lymphosarcoma P-1798.[12]

Recent excellent reviews have been given of the progress concerned with mechanisms of resistance in neoplastic cells.[3, 26, 47]

Several model systems have been developed that have specific advantages for detailed studies of mechanisms of drug resistance in neoplastic cells, including an *in vitro* system developed by Nichol,[26] employing ascitic lines of leukemias in the mouse resistant and sensitive to various antimetabolites. This system lends itself to a study of certain characteristics of resistance to antifolic compounds such as the formation and function of Cofactor F (folinic acid) and its metabolic alterations, the role of the functional forms of folic acid in the biosynthesis of purines, or the interconversion of serine and glycine.

Another system involves certain reactions concerned with the *de novo* synthesis of purine mononucleotides that have been characterized in pigeon liver. These reactions are extremely sensitive in ascitic leukemic cells and have been adapted for the study of inhibition of enzymatic reactions by certain glutamine antagonists in sensitive and resistant populations of cells.[48, 49]

Preliminary findings employing both systems indicate that metabolic differences are measurable *in vitro* by these techniques and that *in vivo* correlations are possible. Further, it is suggested also that the actual differences in concentration of a drug influencing sensitive and resistant leukemic cells are small in contrast to differences commonly observed in bacteria. Accordingly, slight changes in metabolism may be capable of conferring resistance.

Recent studies made by Buchanan and his co-workers deal with individual enzymes and enzymatic reactions such as the inosinic acid transformylase system,[50] the enzymatic reactions concerned with the formylation of glycinamide ribotide,[51] and the characterization of the various substrate and cofactor requirements of the systems. It will be of interest to compare the influence of folic analogues on these individual reactions with which these specific enzymes are concerned in resistant and sensitive neoplasms.

Conclusions

The occurrence of resistance in neoplasms following initial sensitivity has been observed with a variety of carcinostatic agents. The greater significance of this character in neoplasms in contrast to resistance in microorganisms and parasites is stressed.

Resistance to folic analogues in certain lymphocytic neoplasms appears to result from mutation, the drug acting as a selective mechanism. Resistance here is seen to be heritable and stable.

Two common patterns of origin resembling the penicillin and streptomycin patterns in bacteria have been observed. In addition there is some evidence that resistance in certain neoplasms may be unstable; reversions to sensitivity occur. Further biological characterization of resistant populations in a variety of neoplasms is necessary.

Elucidation of the basis of drug resistance in neoplastic cells at the biochemical level can be approached experimentally; several model systems are in use. The possibility of exploiting this knowledge, as well as other basic knowledge concerning such factors as origins, patterns, cross-resistance, and collateral sensitivity characteristics to preclude or circumvent resistance must be considered.

Resistance in certain neoplasms in man, after initial sensitivity, appears by analogy to be similar to that encountered in the experimental animal. Correlations between mouse and human leukemias provide an encouraging basis for obtaining definitive information on this issue. Such studies in man now appear technically feasible.

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Discussion

T. S. Hauschka (*Roswell Park Memorial Institute, Buffalo, N. Y.*): As emphasized by Strong, the somatic mutation hypothesis does not insist on point mutations proper at definite loci as keys to autonomous growth. Nor does it postulate uniform genetic means for achieving diverse neoplastic phenotypes. The popular tacit assumption of a primary metabolic change at the root of all cancer is reinforced by the high aerobic glycolysis of tumor tissue, believed to be the cell's answer to irreversible respiratory damage. However, the vast heterogeneity of neoplasms and carcinogenic stimuli argues against unitary etiological mechanisms.

Each cancer—by whatever agent initiated—is a new biological species with a reproductive advantage over its host. It differs, not only from the organism in which it arose, but from all other tumors. This individuality is best reflected in the cytologic and antigenic features of neoplastic cell populations. While aneuploidy is rare in normal embryonic and adult tissues, the majority of transplantable tumors, spontaneous tumors, and tissue cultures with neoplastic potential are now known to be heteroploid. Like species, they have their characteristic ideograms with distinct chromosome numbers and morphology.

They exhibit also antigenic alterations that can be investigated with greater precision than any other cellular trait. Some normal isoantigens may persist, others are weakened or lost, and some are entirely new. Even two tumors originating in the same tissue of a single mouse of an inbred strain are not alike in their histocompatibility. The isoantigens, of course, have nothing to do with carcinogenesis, but they are convenient test material for demonstrating tumor individuality.

Chromosomal imbalance favoring neoplasia could arise by mitotic mistake in any island of somatic cells made hyperplastic by a carginogen or virus. Gross genetic imbalance seems to be responsible for the high cancer rate in hybrids from crosses between certain species of tobacco, fish, and mice. One function of aneuploidy and of chromosomal mismatching may be the unbuffering of buffered deleterious genes. Accidental separation of growth-controlling genes from corresponding suppressor systems is another possibility. Wherever there is somatic proliferation there is opportunity for unequal chromosome distribution between cells; there is also the menace of losing inhibitory complexes. Genotypic imbalance in favor of growth may thus pass the neoplastic threshold.

This concept does not exclude the possible oncogenic role of true point mutations. However, the gross chromosomal anomalies in tumors are so frequent, and constitute so many novel gene combinations, that they are perhaps not always the empty cart behind the invisible horse. To dismiss the nuclear individuality of most tumors as a meaningless by-product of a fundamental metabolic lesion is no less extreme than Theodor Boveri's intuitive deductions from sea urchins, ascribing *all* cancers to chromosome irregularities.

The somatic mutation hypothesis will survive as one of the most persuasive theories in cancer biology, even though it seems beyond the reach of direct experimental attack. Cytogenetic aberrations in established tumors—no matter how incriminating—cannot be admitted as evidence for a self-perpetuating genetic blunder during the birth of one remote somatic ancestor.

MALIGNANCY AND THE GENETICS OF THE SOMATIC CELL*

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It is a truism by now that the change from the normal to the neoplastic cell must involve a change in cellular heredity. However, this statement is now so general as to be meaningless (compare Burdette, 1955); it demands a rephrasing in concrete terms and a more definite conceptual analysis.

So-called alternatives to changes in cellular heredity. First, let us examine what alternatives there are to changes in cellular heredity. These alternatives are usually proposed by nongeneticists, and some of them are merely the genetic hypothesis specified in terms of a particular phenotype. For example, Warburg's (1956) postulate of a derangement of the respiratory mechanism of the cell is a special case of hypotheses placing emphasis on cytoplasmic factors such as mitochondria and, in more general terms, plasmagenes. Again, let us consider the virus hypothesis (for example, Oberling and Guérin, 1954; and the topic of major emphasis in *Subcellular Particles in the Neoplastic Process*, 1957). This can logically be divided into two categories, one being the case in which the frank tumor cell no longer transmits the virus; in the other case, similar to that of the lysogenic bacteria, the virus is believed to perpetuate itself within the tumor cell and somehow to be responsible for the malignant phenotype. The first case is not really to be distinguished from the mutation hypothesis: here the virus is merely a special mutagenic agent. In the second, the virus has become part of a new cellular heredity, whether by way of incorporating itself into the chromosomal mechanism, or by some less regular mechanism of multiplication in the cytoplasm.

A similar confusion between genotype and phenotype exists in the discussions of an immunological theory of cancer (Green, 1957); the antigens presumably lost in the cancer cell according to this discussion, must also be genetically determined; and we are back where we started.

The examples just given quickly reduce, like others (for example, Caspersson and Santesson, 1942) to special cases of a genetic interpretation of the neoplastic phenomenon. More seriously considered as an alternative concept is the view that the malignant process is an aberration of the process of differentiation (Foulds, 1954; Furth, 1953; Greene, 1955). Implicit in this formulation is a contrast between the mutational processes and those of differentiation, the essential reason for the contrast being the low frequency and the random nature of mutation versus the regular occurrence and the exquisite order of differentiation. Here again, as in the two earlier examples, we are confronted by distinctions that eventually may turn out to be artificial, since they were based on imperfections of analysis. The fact is that ifferent strains of tissue cells do maintain a kind of genetic identity about vhich we know very little at the present time. It was the realization of this act that led Morgan (1934), almost twenty-five years ago, to conclude that he dogma of the genetic equivalence of the nuclei of differentiated cells could ot be accepted, that some sort of irreversible change must occur in cell eredity during differentiation.

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Genetic analysis of somatic cells. It is evident from this capsule treatment hat the genetic concept of the neoplastic process is at the core of the problem: how are the processes of cell heredity changed during this event? The so-called alternatives are merely the result of an inadequate understanding f the nature of the genetics of somatic cells.

What is required to make analysis possible? If the normal tissues from which the neoplasms derive contain many different genetic types, a methodology is necessary that will relate these genetic differences to those with which we are already familiar in the germ cells (Lederberg, 1956; Hauschka, 1957). We must remember here that the essence of the genetic technique is the analysis of the transmission of alternative characters. This is accomplished in classic genetics by the triad of mutation, of fertilization, and of segregation and recombination at meiosis: the mutations give the differences, fertilization gives a nucleus containing the contrasting elements, and the meiotic phenomena allow that the relationships be analyzed to provide the genetic maps.

With the perfection of single cell clonal analysis in tissue cultures (Puck, 1957; Puck and Fisher, 1956), it is now evident that the first requirement—the exact description of cellular differences in sufficient variety to give a large enough number of markers for genetic mapping—can eventually be met. The nature of the cell markers is already varied, from antigenic responses, drug resistance, and nutritional requirement to cell behavior and morphology. The nature of mutational processes in somatic cells is the central problem; we shall return to them later.

Before doing so, let us consider how genetic analysis could be carried out in somatic cells. Fertilization and an accompanying nuclear fusion are difficult processes to accomplish in the soma. The existence of transduction in bacterial clones and the demonstration that virus infection is related to this phenomenon, for lysogenic strains at least (Hartman, 1957; Lederberg, 1957; J. Lederberg and E. Lederberg, 1956), has raised hopes that such possibilities may also exist for somatic cells in multicellular organisms. Let us consider these possibilities more closely.

The process of transduction after the transfer into the cell involves the incorporation of a chromosome fragment into a normal sequence by a process conjectured to be equivalent to the double crossover of classic genetics. There are thus two separate elements in the process, one analogous to fertilization, the other a postzygotic meiosis. It is of some interest that postzygotic meiosis is of common occurrence in microorganisms, but is unknown in higher organisms.

Thus, the essential element of the transductive system is the transfer of part of the nuclear apparatus and its incorporation into a functional chromosome. I should like to recall here extensive trials, particularly those attempted in maize, to determine whether fragments of chromosomes withou a centromere could be reincorporated into the genome. The experiments involve an apparently unrelated field: the study of the mechanisms of production of chromosome rearrangements. Two apparently opposing theories were being tested (for general background see Muller, 1954): on the one hand it was believed that fragments of chromosomes recombined to give the viable rearrangements while, on the other, it was believed that exchanges occurred between nonhomologous regions with no prior breakage. For a time, early in the history of the work, the existence of small patches of the original type of tissue in mutant kernels of maize endosperm was regarded as evidence that chromosome fragments, after a series of mitoses in which they did not replicate, could become incorporated into the mitotic apparatus and become part of the genome. These recovery spots, as Stadler (1930) called them proved to have a different interpretation on the basis of McClintock's studies (1938a, b; 1956) of different kinds of variegation: they are apparently the residue of the original cell type after a process of loss has occurred early in development. In point of fact, there is no evidence that acentric fragments will become permanently incorporated into the nucleus; they seem generally to lag outside the spindle and to be lost in the cytoplasm. Even when occasionally carried in at telophase (McClintock, 1938a), they are lost in successive divisions, a condition not at all hopeful from the point of view of transduction. Tests for the occurrence of crossovers from acentric fragments, the essential point in the application of the transduction argument, would be highly desirable.

There have been some claims for the occurrence of transformationlike phenomena in vertebrates; namely, genetic transfer from DNA fractions as in bacteria (Hotchkiss, 1957). One is the leukemia case (Stasney *et al.*, 1950); the other, the more recently publicized experiments in ducks (Benoit *et al.*, 1957). As to the leukemias, the known virus etiology of such malignancies makes them a special case even if the data are accepted at face value, and even if the critical experiments of Klein (1952) with genetically marked material are regarded as merely cautionary as to the possibility of transmission of whole cells. As for the duck, it is difficult to be convinced by the information so far available.

It would undoubtedly be folly to exclude the possibility of transduction processes in the analysis of somatic cells. However, it would also be unrealistic to paint a picture in which transduction in metazoa were to figure as a possibility wholly untested, merely awaiting proper experimental conditions to provide the proper analytic approach to our problems.

As an alternative to transduction, the fusion of cells with the subsequent union of nuclei to form a tetraploid nucleus may be considered. This is a phenomenon long discussed as an origin of tetraploidy. In recent years however, it has been relegated to a minor position as the processes of endomitosis have been clarified (Geitler, 1953). Nevertheless, the possibility of such a technique lies open.

Recombination in such a system would also involve crossing over, now o a mitotic type. This is an event well known in *Drosophila* (Stern, 1936), and in certain microorganisms, of which *Aspergillus* is the most extensively studied (Pontecorvo, 1953). It is still not clear in either of these organisms what the relationship is between the mitotic and the meiotic crossing over. In *Drosophila*, for example, intimate association between the homologues, a necessary prerequisite to recombination, is characteristic of every somatic prophase; the unpleasant fact here is the rarity of somatic recombination.

Nuclear transplantation and genetic analysis. Let us consider still another approach to this problem. What we really want to test, as a general proposition, are the possible differences in genetic composition between different types of tissue cells, the differences that cause them to maintain their identity and that contain, as a special case, the characteristic differences of the malignant cell. Infectious processes, the inheritance of cytoplasmic particles, the differentiation of nuclei—the analysis of all of these necessitates techniques that will allow the substitution of one cell component for another. Conceptually, the simplest of these techniques for somatic cells is nuclear transplantation. I should like to discuss now the possibilities that this procedure offers, as Briggs and King (1955) and King and Briggs (1956) have developed it in the frog's egg.

Let me recall their experimental procedure in outline: into an egg from which the polar bodies and the female pronucleus have been removed, at an appropriate time after activation, there is injected the nucleus (with what cytoplasm adheres to it) from a previously isolated cell at one of the stages of embryogeny. They have been able to show, in a series of elegant and well-documented experiments that, through the blastula stage of development, nuclei, when transplanted, will produce normal embryos. In agreement with the classic experiments that overthrew the Roux-Weissman theory, there is no specialization of the nuclei in the frog at this time. As development proceeds, however, the picture changes, and a new prospect opens. The most elaborate series of experiments, involving several nuclear-transplant generations, concerns the behavior of endoderm nuclei at early stages of germ-layer segregation. The evidence is clear that embryos developing from such transplanted endoderm nuclei show a deficiency of ectodermal structures. It is obviously too early to say whether the indications of specific restrictions in potentiality will be fulfilled; but the fact that it is the ectodermal structures that are inhibited is, to say the least, suggestive. One may conceive of an irreversible change of chromosome constitution, of such nature that only special regions transfer information in the different specialized cells, the rest being silent. This point of view has repercussions for the problem of neoplasia, as I shall attempt to show. However, what concerns us here is the possibility of developing a methodology for mapping somatic chromosomes on the basis of these discoveries.

I should like to mention two possible experimental approaches to this problem, both difficult to accomplish. One is the study of the progression of specialization in development to determine the sequence of changes in nuclear differentiation. In this way the changes during development could be mapped with respect to each other, and a beginning, however laborious, might be made toward their analysis. The other possible approach involves the normal fertilization of the egg by the sperm, the removal of the female pronucleus, and the introduction of a differentiated nucleus at the usual time for transplantation. Here a triploid zygote nucleus should be formed by the fusion of the sperm and the transplanted nucleus. The opportunity would then be clear for a conventional genetic study of the triploid zygote, heterozygous for differentiated and undifferentiated chromosomes. This would be a straightforward analysis, if feasible, and I mention the possibility simply to show that the analysis is not completely out of bounds.

Possible genetic nature of differences between cell types. I now return to the possible kinds of genetic differences between cells, in an attempt to evaluate their respective importances, first with respect to ordinary tissue differentiation and then with regard to the malignant process itself.

If there is perhaps any one outstanding thing that genetic studies have impressed upon us, it is the variety of hereditary changes that may be responsible for a given phenotype. The genetic system is a balanced one, and its disruption in any one of many ways will lead to a mutant character. Any organism at all well studied genetically, from *Escherichia coli* to *Drosophila*, from the mouse to man, shows examples (for general background see Wagner and Mitchell, 1955) of mutations at different loci in the chromosomes that have similar end effects. Moreover, simple quantitative changes—for example polyploidy, chromosome duplications or deficiencies—may give distinctive mutant phenotypes: the original mutations of DeVries belonged to this category. This is a point that needs no laboring.

The relevant questions, however, are the relation among the known types of mutation in the germ cells as studied by the usual genetic techniques, the special types of somatic mutation that have yielded to analysis, the differences between tissue cells, and the malignant process. Somatic cells are known to respond, on the whole, to the same mutagens as the germ cells; the rates of change per individual gene, where these have been measured, are of the same order of magnitude in both kinds of cell. However, these are rare changes, and there are types of somatic mutation that occur with a high frequency. These types form the variegated races of both plants and animals.

In *Drosophila*, particularly in *Drosophila melanogaster*, where these problems have been studied in some detail, there are, broadly speaking, two kinds of somatic variegation. One is associated with somatic crossing over; the other, more like a mutational event, is associated with a special type of chromosome rearrangement in which the position of the genes with respect to the so-called heterochromatic regions determines their behavior (Hannah, 1951; Lewis, 1950; and Schultz, 1939). The two types of variegation (somatic crossing over and heterochromatin sensitive) are different in their response to metabolic antagonists of various types. From the present evidence it would be somewhat surprising if these types were related phenomena, the frequency of somatic crossing over being influenced by the fact that substances such as the nitrogen mustards have a relation to chromosome rearrangement, the heterochromatin-sensitive variegation responding to substances generically having to do with nucleic acid synthesis (Schultz, 1956).

The heterochromatin-sensitive variegation is a complex genetic phenomenon. It depends on a constellation of factors; the patches of mutant tissue appear in individuals of the proper constitution when a locus is placed next to a heterochromatic chromosome region by means of the chromosome rearrangement. The size and number of the patches and the type of mutant allele displayed depend on: the distance of the locus from the point of rearrangement, the kind of heterochromatic region, the quantitative relations of other heterochromatic regions in the chromosome complement, and a galaxy of modifiers distributed through the chromosomes. Moreover, the influence of these factors, at least in part, is exerted differentially at different stages of development: the effects are maximal at the later stages, but are not detectable in tissues differentiating early, such as the gonads. As a corollary to this fact, loci more distant from the heterochromatic regions of the rearrangement are affected only in the later stages; the closest loci are affected in the first cell divisions of the series in histogenesis, those more distant in the later ones. These relations emerge clearly from a study of the patterns of variegation in properly marked individuals. It is possible to say, from a series of studies carried out for this purpose, that the change occurs at the stage of the cell cycle during which the nucleus is differentiated; not thereafter, when the growth of the metabolic nucleus occurs (Schultz, 1956).

The cytological and cytochemical analyses of these phenomena have been interesting. In the giant chromosomes of the salivary glands, the sensitive regions show a change in structure, from the banding characteristic of their normal site to a meshwork like that of the heterochromatic regions themselves. In more extreme manifestations, the affected region is no longer recognizable as such; in a less extreme case the normally light bands became darker, constituting evidence of an increased content of DNA. The change in genetic activity is thus associated with a change in the chromosome (Caspersson and Schultz, 1938; Schultz, 1952, 1956).

It was this series of observations, made many years ago, that placed emphasis first on the concepts that the nucleic acids had genetic importance and that the heterochromatic regions of the chromosomes were important in controlling the synthesis of the nucleic acids. More recent evidence has strengthened these hypotheses: the heterochromatic Y chromosome, which has a potent influence on variegation, has been shown to affect the kind of ribonucleic acid in the *Drosophila* egg, as well as the constitution of the pool of nucleic acid precursors. The evidence indicates that the effect may be at the level of the synthesis of the bases themselves (Levenbook *et al.*, 1958; Schultz, 1956; Travaglini *et al.*, 1958).

From another direction, the study of the effects of antagonists to nucleic acid synthesis on the manifestation of variegation has also given supporting evidence (Schultz, 1956). The most extensive results are those with the antifolic substances such as A-Methopterin. Here there are pronounced effects on morphogenesis as well. For the bristles of the fly, the effect on morphogenesis can be compared directly with that on the variegation change.

Both these effects of A-Methopterin—on the number of bristles and the frequency of the variegational change in the shape of the mutant bristle—can be reversed by thymidine, and other experiments show that the step involved is the synthesis of thymidine from desoxyuridine. Thus, one has a picture of a tissue in which thymidine synthesis is limiting for DNA formation and the DNA synthesis is critical both for morphogenesis and for genetic specificity in the formation of bristles. The situation is still more complex, however, since the thresholds for the effect on bristle number are different in different regions of the body, and the effect on the variegation may even be negatively correlated with the morphologic effect. That is, the regions in which the increase of bristle number is greatest are those in which the effect on variegation is quite slight.

This situation, to my knowledge, is the closest approach yet made to a simultaneous study of change in genetic specificity and the processes of morphogenesis. In this case both are DNA determined, and yet the impression grows upon one that different systems are involved. A possible hypothesis might be expressed as follows: the slowing of DNA synthesis beneath a certain level is somehow concerned with the morphogenetic effects; but when this level has been reached, the competitive relations at the site of the affected genetic locus are such that a normal DNA can now be synthesized, and a bristle of the typical shape for the strain be formed.

There are a number of implications here for the malignant process, which I shall examine later: the interrelations in nucleic acid synthesis, with their repercussions on the genetic specificities in tissue cells; the mutation pattern with its specificities for the different tissues, easily altered by modifiers in the heterochromatic balance. However, I now turn briefly to another system, brilliantly investigated in maize (McClintock, 1956) and probably general for the plant kingdom, which also shows the existence of factors controlling the behavior and activities of other loci, with apparent effects of position also, but with an additional feature not present in the *Drosophila* variegation, namely, a high frequency of transposition of these elements. I refer to the Activator-Dissociator complex studied by McClintock.

The importance of this analysis for this discussion is that, like the *Drosophila* variegation, it shows the existence of a dual relationship of the controlling elements to the mutational process. One factor is the local rearrangement: namely, the heterochromatic break in *Drosophila* and the so-called Dissociator locus in maize. The other is the so-called Activator, the factor responsible for tipping the balance in the nucleus as a whole in maize—in *Drosophila* the Y-chromosome—and in other heterochromatic regions. The analysis in maize has not yet reached the point at which the chemical steps can be defined. However, it would not be surprising if the elegant maize data also fitted into the concepts developed for *Drosophila*, of regions concerned with the nucleic acid metabolism of the chromosomes controlling the specificities displayed by the other loci of the complex. The difference in detail, so striking as to be almost a difference in kind, serves to outline the range and variety possible among these systems. With this background briefly sketched, it is evident that the patterns for genetic susceptibility to tumors might very easily be accommodated within a framework of a genetic control of somatic mutation.

By way of resumé, before entering on the application of these points of view to the malignant process, let me restate them. The genetic mechanisms for achieving a change of phenotype in a somatic cell are many: aneuploidy, doing violence to proper genic balance; chromosome rearrangements, with the possibility of (1) stable position effects at the points of rearrangements, (2) successive "mutations" extending along the chromosome, as in variegation, and (3) transpositions of controlling elements leading to still new position effects at successive cell divisions. These are nuclear events. They control cytoplasmic changes of phenotype, some of which may persist in the cellular heredity of a clone, until conditions of stress excite a change to an alternative state. One example to be mentioned here is the Sonneborn analysis of ciliary antigens in *Paramecium* (Beale, 1957; Nanney, 1957); another, the all-or-none enzyme induction in *E. coli* under conditions of low substrate concentration, shown by Novick and Weiner (1957).

In addition to the nuclear events we must leave open the possibility of cytoplasmic particles, viruslike infective agents. Even here the relationship to nuclear elements is close, as the study of lysogenic strains of bacteria has shown. The prophage has a locus in the bacterial chromosome (Hartman, 1957; Jacob and Wollman, 1957). It is of some consequence that the stresses that cause the change from the prophage in the bacterial chromosomes to the mature virus are generally competent to cause chromosome breakages in multicellular organisms. From this kind of consideration, it follows that there is no real contradiction in essence between the so-called virus theory and the genetic concept of cancer. The problem to solve is the genetic analysis—with the experimental challenge of inducing nuclear fusion and the equivalent of meiosis in somatic cells or of devising techniques adapted from the nuclear transplantation experiments to give true genetic analyses—two procedures that are not necessarily alternatives.

The application of these concepts to the malignant cell is almost a trite exercise. The phenotype of the malignant cell, particularly in vertebrates, is distinctive and yet, in an odd way, nondescript (for reviews see Huxley, 1956; Klein, 1956). Having lost the characteristics of the specialized tissue from which it originated, it converges on a cell type like that of the embryonic cell but different, in its potentialities, in the kinds of information to which it can respond. The development of this phenotype is a gradual one: this gradual nature is discussed elsewhere in these pages by Strong; it has been emphasized by Foulds (1954) and by Furth (1953) and is contained in the discussions originating with Rous and Beard (1935) and emphasized by Berenblum (for example, 1956) of the initiating and promoting phases in carcinogenesis. The tumor progression has been likened to differentiation—the original tumor cell being dependent for its growth on whatever specific substances, such as hormones, that its normal cell type demanded; proceeding from this conditioned growth, in Furth's phrase, to an ever more autonomous state. It is of considerable consequence that this progression, as far as growth requirements go, is not correlated with the malignancy of the tumor.

As Foulds himself says, one deals with unit characters. However, once having stated the case in this way, one must continue in the framework of conventional somatic mutations and selection, about which other contributors to this publication have much to say: and even here still obscure "adaptive" mechanisms also exist (Klein and Klein, 1957).

The key problem is the original change, the incitement to continued cell division. Once this is given, conventional theory can take over. However, what is this original change? A specialized cell, under a great variety of conditions, some special for the particular cell type (butter yellow in the liver, for example) loses some, but not all, of its specialities, and forms a focus of malignancy. The cytological descriptions of the process are not too clear to me: the exact interrelations of nuclear and cytoplasmic phenomena have not been worked out in unquestionable detail, insofar as I can see (for a discussion, see Biesele *et al.*, 1956). Much has been made of the mutagenic behavior of carcinogens, but these also affect cytoplasmic structures, and the initial effects as studied by such means as fluorescence microscopy would seem to be on such structures as mitochondria, which break down. What remains is the chromosome complement, sometimes polyploid, due to endoreduplication operating in an abnormal cytoplasmic environment.

It has been my belief for some years that the nature of the original change could not be determined until more was known of the properties of the chromosomes in the cells that became malignant. Formally, all can be accounted for by mutational changes of one sort or another. Actually, however, it is important to know what we start with in the normal differentiated cell. The beginning that we now have in the experiments of Briggs and King (1955; King and Briggs, 1956) tells us that changes have occurred, and that these may be of a nature related to the restrictions of potentialities in the differentiated cell. Some years ago I discussed a kind of nuclear differentiation that might account both for the normal specialization and for the malignant process (Schultz, 1947), although there was no experimental evidence for it. The results of King and Briggs encourage this line of speculation.

What I had supposed was that the kinds of change apparent in the heterochromatin-sensitive variegation, local changes in genetic specificity, could be related to the interrelated yet contrasting properties of the genes, namely, their replication and their role in specific cellular synthesis. Both involve nucleic acid metabolism, but a slight alteration in the DNA, for example, could restrict the activity to replication. It is this type of difference that I conceived of as happening during development, and as distinguishing the heterochromatic regions themselves. It is a primitive speculation, but I should like to follow its consequences after considering some of the exciting cytogenetic data obtained on tumor cells in recent years.

One of the remarkable features of the malignant phenotype, noticed almost as soon as the techniques of observation were adequate, has been the high frequency of cells with abnormal chromosome numbers (Koller, 1947). It was this that led Boveri (1912) originally to propose that the malignant phenotype resulted from a change in genic balance by way of aneuploidy.

The question has always recurred whether the aberrant chromosome numbers were cause or consequence of the malignant change; the ways in which the question can be argued are many, and I shall not take time to rehearse them. As a result of the work of Hauschka and Levan between 1953 and 1956 (Levan and Hauschka, 1953; Hauschka, 1953, 1957), of Ford *et al.* (1957), of Makino (1956) and his group, of Yerganian (1956), of Koller (1956) and of Hsu and Morehead (1957) and Hsu *et al.* (1957), evidence is now accumulating that a truly euploid malignant cell may not exist. Ford is of the opinion that each of the primary tumors he has examined, like the clones of Hauschka and Levan and the stemlines of Makino, have a characteristic aneuploid karyotype.

To this another series of facts should be added, namely, the high frequency of rearrangements that must occur in tumor cells, according to the work of Levan (1956a, b) in mouse and man and, according to Yerganian (1956), in the hamster.

What is really striking here is that there seems as yet to be no characteristic aneuploid number for the neoplastic cell type. What does this mean? There are a number of possibilities, some deriving from orthodox genetic theory. It is quite possible that any one of a large number of genetic changes may be adequate to trigger the malignant reaction, and therefore the chromosome variability appears as an epiphenomenon, part of the phenotype of this kind of cell. Each clonal group has its own stemline, as has been postulated by many workers, and the variability around this is important for the further behavior of the population. In this case, the diversity of malignant stemlines must mean, as already said, that many kinds of genetic change are adequate; or that, when the particular change occurs, it is insensitive to modification by other genes.

These are formal explanations and, in a way, they seem to me to prove too much. The chromosomes of tumor cells behave almost as if they were indifferent genetically. This degree of aneuploidy in the germ cells of any of the organisms studied genetically would be catastrophic if the chromosomes had their accustomed roles. In the tumor cell, it is almost as if the chromosomes have, as their main function, their own replication.

I now return to the speculations concerning the genetic constitution of differentiated nuclei. What I had postulated was that, in these differentiated nuclei, the genetic material undergoes a change, restricting the transmission of information, the possibility of specific genic activity, to only a portion of the complement required for the functioning of the specialized cell type. The constitution—presumably of the DNA—in the remainder permits replication, but functions now in what we may call, for lack of information, a generalized way. The model for this kind of speculation is the heterochromatic chromosome with little genetic specificity of the usual kind, but a function in nucleic acid synthesis (Schultz, 1956). What is to be expected when such a cell is stimulated to divide under the special circumstances that obtain during carcinogenesis: damage to the normal cytoplasmic apparatus also? Under these conditions, the growth of the daughter cells is of a new kind: the competitive relations of the different loci in the nucleus are changed in comparison to what they were during the original differentiation of the cell type. The lack of responsiveness to the stimuli for differentiation with, at the same time, retention of the specific needs of the cell type of origin would follow the postulate of nuclear differentiation. These characteristics could give rise to some of the aspects of the malignant phenotype. In particular, the behavior of the chromosomes and their apparent heterochromatic character would be quite consonant with such a hypothesis. How may we test it further?

The reality of these considerations depends on the existence and the nature of nuclear differentiation in development. The ideal test would be a comparison of the behavior of cultures of aneuploid clones of cells from the blastula, with similar aneuploids from later stages of development. In this way a possible evaluation of the activities of the same chromosome from different stages of development might eventually be obtained. The nature of the changes, in terms of chromosome constitution, is quite another problem. If these changes, as I have been inclined to believe, are related to the nucleic acid composition of the chromosomes in a way similar to the sensitivity of some variegation strains to thymidine deficiency (Schultz, 1956) a direction of research is evident.

This is all a far cry from the simplistic antitheses of virus etiology, or somatic mutation, or respiratory damage, or other such factors. The situation is complex, as complex as the developmental system from which the malignant cell originates and on which it parasitizes. I do not believe that these complexities will be so polite as to disappear if we ignore them; they are more likely to baffle us, with all the perversity that animate nature can add to the inanimate. Moreover, these complexities are, in a way, what make the problems of malignancy so fascinating also for general biology, for they constitute a challenge to find the principles that resolve the complex into the simple.

Summary

Almost by definition, the malignant cell differs from the cell of origin by a change in its cellular heredity. This paper has been concerned with an examination of the general principles thus far established for changes in somatic cells and with attempts to evaluate their relevancy to the malignant change.

The malignant tissue is characterized by cells that have a definite reproductive advantage and that migrate easily to different sites within the organism that they parasitize. The malignant phenotype is quite diverse within this scope, different tumors maintaining characteristic resemblances (including genetic markers manifest in those cells) to their tissue of origin. The appearance of malignancy is gradual ("progression"), during a series of cellular reproductions which give opportunity for the selection of more malignant cell types (for example, "conditioned" versus "autonomous" growth). Malignant cells present a phenotype of chromosomal instability not unfamiliar to students of the genotypic control of chromosome behavior. Since no one aberrant chromosome number is characteristic of a malignant

cell, and since many aberrant chromosome types are found in malignant cell populations, it follows that the malignant phenotype is controlled by many chromosomal loci that interact, or that the malignant reaction is relatively insensitive to changes in chromosome number, or that the chromosomes of malignant cells are relatively indifferent in their genetic content.

The variety of possible or probable genetic mechanisms to account for these phenomena is considerable: quantitative changes in the balance of chromosomes or parts thereof, due to nondisjunction, polyploidy, or chromosome rearrangement; changes in genic constitution proper, resulting from somatic crossing over or point mutation; virus infection, with resultant changes in phenotype in the host cell; transduction or other phenomena of chromosomal transfer from cell to cell; and finally, the now serious possibility of irreversible genetic change (nuclear differentiation) in somatic cells during normal development. The interdependence of somatic mutation of certain types and the differentiation of tissues is exemplified in a discussion of variegation phenomena, and the relation of these phenomena to the nucleic acid metabolism of the cell is indicated. These diverse phenomena are considered in examining the possibility of criteria by which they may be distinguished from each other in the events leading to malignancy. In this discussion the possibility of nuclear differentiation must take precedence, since most malignant tumors originate after histogenesis, that is, from cells in which nuclear differentiation has probably taken place. It now appears that the problem of the comparison of malignant with early embryonic cells recurs at the level of the nature of the chromosomes themselves, and that relations observed in the variegation process may offer a useful guide in exploring mechanisms for chromosomal differentiation by way of the constitution of the nucleic acids concerned with heredity.

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GENETIC STUDIES ON TUMOR IMMUNITY: AN ANALYSIS OF C3H SUBLINES AND THEIR TUMORS*

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Although there is abundant evidence that genetic factors play a part in the etiology of cancer, nothing is known about the mutations generally thought to be responsible for the change to malignancy. Experience gained from transplantation has indicated that progressive simplification of graft requirements occur;[1] this has long been taken as evidence for mutation in a transplanted tumor. More recently, chemical, cytological, and immunological methods have been used in attempts to categorize differences between malignant and normal somatic cells.

A hint that mutations occur very early in the history of a tumor is given by Foley's[2] experiments with methylcholanthrene-induced sarcomas, repeated by Baldwin.[3] This work was recently extended by Prehn and Main.[4] Animals in which the original tumor was removed were resistant to subsequent inoculation with the same tumor, and cross immunity was demonstrated between different methylcholanthrene-induced tumors. It also appears from the figures published by these authors that the induced tumors transplanted less consistently than did either skin or fibrosarcomas of spontaneous origin, underlining the suggestion of inherent incompatibility between graft and host.

One of the few methods at present available for a precise study of somatic genetics is that of determining the antigenic pattern of tumors and of the corresponding host strain. An extension of this study is strongly indicated in view of evidence of antigenic differences between graft and host gained from transplantation[2] and from transference of immunity.[5] For purposes of this present investigation, Strong's C3H and its related strains and sublines have been used.

The first procedure was to investigate the one antigenic system that has been worked out in any detail. This is the H-2 system, for which linkage relations were established by Snell and his colleagues[6, 7] and the serology of which has been unraveled largely through the careful work of P. A. Gorer. The H-2 antigens are the product of the most potent of the histocompatibility systems in the mouse.

First called "histocompatibility locus 2" because it appeared to coincide with Gorer's antigen II previously demonstrated in rabbit antimouse serum, it was thought to consist of only two factors, D and K, both of which were present in A-strain mice.

As a result of the work of Gorer, G. F. Hoecker, and their colleagues, and following the development of an improved method for detecting isoantibodies, it was established that a much larger number of factors was involved. For

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some time, 5 factors were known on the A-strain cell, with evidence for the existence of multiple allelism for 2 of them[8] and with an additional 3 antigens, Q, P, and S,[9] which could have been allelic to D or K.

More recently Hoecker has demonstrated 3 more antigens, G, H, and I.[10] The H antigen occurs on A cells, bringing the number of factors to 6, and we have detected several more, bringing the number up to 10 or possibly more.

Several observations of crossing over within the H-2 group of antigens[8, 11] show that the controlling genes are spatially separated; H-2 appears to consist of a number of closely linked genes with a measurable cross-over frequency between the more widely separated members of the complex. Gorer has estimated the cross-over frequency between D and K to be about 1 per cent but, because of the complexity of the system, such estimates of cross-over frequency are likely to be low rather than high. The occurrence of individual antigens in the lines derived from the original cross-over mice is being determined, and their relative positions in the region worked out. The distribution of some of the H-2 antigens in some of the mouse strains used in this study is shown in TABLE 1.

TABLE 1
SOME OF THE H-2 ANTIGENS OF FIVE COMMON PHENOTYPES

Strain	Phenotype	Antigens
A/He Ha	H-2a	A. C. D. E. F. H. K. M. N.
C3H/St	H-2k	A. C. d. E. f. H. K. m. n.
C/St, DBA/1	H-2q	a. C. d. E. F. h. Q. M. n.
BALB/C, DBA/2	H-2d	a. C. D. E.^{d}. F. H. k. M. N.
C57BL/Ha	H-2b	a. c. D.^{b}. E. F. h. k. m. N.

Of the most recently detected antibodies, those against L, M, and possibly V are the most strongly reacting. Anti-A appears to be rather inconstant in its appearance, but it reacts strongly when present; anti-N appears to be weakly reacting and is not always present. Linkage with known H-2 antigens has been shown for these factors in backcross tests. A more complete description will appear elsewhere.

Antigens of the Mice

The C3H family, with its numerous sublines, was chosen for this study, since the different lines of descent were known to vary considerably, not only in their response to transplanted tumors, but also in certain skeletal characters, in tumor incidence, and in some biochemical characters. They were also known to be closely related to the Bagg, A, and DBA strains. For simplicity in the text, the mice are referred to only by the name of the strain, except where different sublines of the same strain were used. A description of the origin of the mice used is given in TABLE 2. Mice not included in the table were all obtained from L. W. Law.

The origins of the A and C3H mice have been described by Strong.[12-14] The lines were originally bred to provide information on spontaneous tumor incidence, and part of the reason for inbreeding was to facilitate transplantation and thereby aid the study of the tumors arising in the stocks. At first there was no separation into lines or strains, but these were later split off at intervals and distributed to other investigators. Differences in coat color show that a process of segregation was still going on at the time the lines diverged—C is cinnamon, C12I was dilute, the other surviving lines are wild type (intense black agouti)—and, from consideration of the H-2 phenotypes of the present-day representatives, it is apparent that segregation for H-2 factors also occurred. It is extremely probable that, even after the extant lines CBA, C3H, CHI, and C had been established, a number of loci were still heterogeneous, and that segregation was still continuing for a number of factors, including those determining histocompatibility.

TABLE 2
THE DERIVATION OF MOUSE STRAINS USED

Strain	Carried by	Generation*	Origin†	Comments
DBA/1	Mirand		Sn → Wa → Mirand	
DBA/2	Hauschka	21	Li → Lankenau → Ha	Little's original Dbr Behaves as DBA/2
YBR	Hauschka	19	Li → He → Wi → Ha	Maintained as YBR-$A^y a$ & YBR-aa
C3H/He	Hauschka	23	St → An → He → U.G.‡ → Ha	C3Hf/He
C3H/St	Hauschka	22	St → Ha	
C58	Hauschka	22	Md → Lankenau → Ha	
129	Hauschka	18	Rr → Ambrus → Ha	
C57BL	Hauschka	22	Li → Lankenau → Ha	Resembles C57BL/6
A/Ha	Hauschka	24	St → Bi → He → Ha	
A/St	Hauschka	19	St → Ha	
F	Strong		St	
I	Strong		St	
JK	Strong		St	
N	Strong		St	
C3H	Strong		St	
C3H.B	Strong		St	
CHI	Strong		St	
CBA	Strong		St	

* Number of inbred generations carried by investigator.
† Symbols for investigators are from *Cancer Research*.[37]
‡ Ufford Green.

CBA and CHI were tested against a number of sera and gave the same general reactions as C3H, while C appeared to fall into the same category as DBA/1. A summary of some of the reactions is given in TABLE 3. The general type of the antibody is presented here, but it is possible that some sera include antibodies against other H-2 antigens. From the good agreement given between the different lines with the same sera, it is obvious that the CBA and CHI react like C3H as H-2k, while C reacts like DBA/1 as H-2q. In these tests there is no evidence of mutation, with one possible exception: the reaction of C with the serum labeled "anti-KH." Preliminary genetic tests indicate that this is an H-2 factor; this would be strong evidence for a true mutation. Further samples of serum are being prepared and will be tested more extensively. If the finding is confirmed it will be made the subject of a separate report.

Most of the sera used in TABLE 3 were prepared by Snell or Hoecker at Bar Harbor, Me., in I.R. lines, and both these and the other sera have been tested genetically to confirm that they do define H-2 antigens.

During preliminary testing it was found that a number of sera would differentiate between two sublines of C3H carried in our laboratory (TABLE 4). These were the C3H/St obtained by T. S. Hauschka from Strong in 1950 in its seventy-sixth generation, and now in its twenty-second generation in our colony, and the C3H/He obtained from W. Heston via Ufford Green by Hauschka in 1949, and now in our twenty-third generation.

These two sublines have been tested with about 65 sera, of which 8 were produced in isoresistant pairs and have been used primarily for reference purposes. In no instance was a reaction obtained with C3H/St cells that was not also given by C3H/He, but 7 sera reacted with C3H/He and not with C3H/St. Of these, 7 were formed in C3H/St against A, C57, DBA/2, or C58. The others were prepared against C57BL in 129, in the F1 hybrids (DBA/1 × DBA/2) or (C3H/St × DBA/2).

For one additional serum, DBA/1 anti-A after absorption with C3H/St tissue, it was possible also to show residual hemaglutinins for C3H/He.

These eight sera all agglutinate C3H/He cells in the absence of any known component against H-2k, and backcross tests completed thus far show that agglutinins not linked to H-2 are segregating. To avoid confusion with the terminology used for the other histocompatibility factors in the mouse, it is proposed to call these factors alpha, beta, etc., until such time as linkage relations have been established, when a conventional nomenclature can be substituted.

Beta is demonstrated by the antibody DBA/1 anti-A after absorption with C3H/St. It is present on C, I, F, A/Ha, YBR, and C3H/He cells, and has been conclusively shown to be a non-H-2 component in a series of genetic tests including absorption with tissue from backcross animals. It is highly probable that beta is also present in the serum C3H/St anti-A, and the same strain distribution of antigens is observed (TABLE 5). This antigen is probably not present in DBA/2, or two components are involved; one of these is absent from DBA/2.

Antigamma is present in the antibody C3H/St anti-C58, which has been shown to have a component separate from H-2 in backcross tests. The same antibody is probably present in C3H/St anti-DBA/2, although linkage tests are not completed; this appears to be a widely distributed antigen, as seen from the table.

TABLE 3
SOME REACTIONS OF MEMBERS OF THE C3H FAMILY WITH VARIOUS ISOANTIBODIES

	Anti-Q*	Anti-Q,E*	Anti-D,H*	Anti-K,H	Anti-K	Anti-D*	Anti-D,F*	Anti-B*	Anti-C,D,H
CBA	−	+++	NT	+++	+++	−	−	−	++
CHI	−	+++	+	+++	+++	−	−	−	++
C3H/St	−	+++	+	+++	+++	−	−	−	++
C	+++	+++	−	+	−	−	+++	−	++
DBA/1	+++	+++	−	−	−	−	+++	−	++

Symbols: * = serum produced in isoresistant lines at Bar Harbor; +++ = strong reaction; ++ = moderately strong reaction; + = weak reaction; − = negative reaction.

TABLE 4
ANOMOLOUS REACTIONS OF SOME H-2k LINES

	C3H/St Anti-A	C3H/St Anti-C57	C3H/He Anti-DBA/2	C3H/He Anti-C57
C3H/St	−	−	−	−
C3H.B/St	−	−	−	−
CBA/St	−	−	−	NT
C3H/He	++	++	−	−
CHI/St	++	++	−	NT
C3H/Fg	−	−	+	++

Symbol: NT = not tested.

TABLE 5
PROBABLE DISTRIBUTION OF FOUR NON-H-2 ANTIGENS

Antigen	DBA/1	DBA/2	Y/He	C3H/St	C3H/He	C58	129	C57BL	A	C/St
Alpha....	−	−	+	?	−	−	+	−	−	+
Beta.....	−	−	+	−	+	+	−	−	+	+
Gamma..	?	+	+	−	+	+	+	+	+	NT
Delta....	−	−	+	−	+	+	NT	+	?	NT

Symbols: NT = not tested; ? = doubtful reaction.

Delta has been shown by its reaction with a weak antibody in the serum (C3H/St × DBA/2) anti-C57. It does not segregate with the H-2 antigens, and it occurs on C57, C3H/He, and A/Ha, but not on DBA/1, DBA/2, I, N, or JK. The same antibody probably occurs in C3H/St anti-C57, and in the antibody (DBA/1 × DBA/2) anti-C57, where it has been shown also to occur on C58 and YBR cells.

The final analysis of these antigens can be made only by further absorptions with four classes of backcross mice, segregating both for H-2 and for the unknown factor, and by testing for linkage with other known markers. The "isogenic" difference between the C3H lines makes it unnecessary to establish separate inbred lines. The reactions obtained against these antigens appear to be different from those obtained with antibodies against H-1 and H-3, where the reactions obtained were extremely feeble. The analysis of these factors is complex, and this must be regarded as a tentative scheme rather than a final classification, but it is expected that the complexity will increase rather than diminish.

Next, some of the serologic reactions of all the readily available relatives of Strong's C3H were tested, some of the animals being obtained directly from Strong, and others through Law. Besides CBA, CHI, and the C3H sublines already tested, C3H.B/St (black-eyed white), C3H/Lw, C3H.E/Lw, C3Hf/G, and C3H/Fg all reacted as H-2k.

As differences had been detected between the Strong and Heston sublines of C3H by the use of C3H/St anti-A, some of the other strains were tested with this serum and with C3H/St anti-C57BL, with antibody produced in H-2k homozygous (C3H/He × DBA/2) × C3H/He (essentially C3H/He) anti-DBA/2, and with C3H/He anti-C57. The reactions are shown in TABLE 4. CHI shares at least one of the antigens described in the previous section with C3H/He, while CBA and the white-haired mutant resemble C3H/St. C3H/Fg appears to be in a special class. Beside the reactions shown, it participates in a number of other reactions in which the other C3H lines do not. No attempt has been made as yet to analyze the antigens present in C3H/Fg.

M. Runner (personal communication, 1957) has examined many of these strains for frequency of eleven skeletal abnormalities; there does not appear to be any correlation between the distribution of any of these skeletal characters and the antigens shared with A.

The analysis of the serologic factors involved has led to a partial unfolding of a fairly complex situation. Backcross tests have shown that, with the possible exception of one very weak factor on C3H/Fg, the other factors are segregating independently of H-2.

To summarize our findings with the mice, the phenotype H-2k is widely distributed among the C3H family and appears to have remained stable despite 90 generations of separation from CBA. A number of independently segregating factors have been detected; two of these could have been introduced from the ancestral Bagg, and the third from the ancestral DBA.

Antigenicity of the C3H Tumors

At least three antibodies of entirely different specificity are likely to be formed following hyperimmunization. These are directed against H-2, against histocompatibility loci other than H-2, and against the X type of isoantigens, which are limited to tumors.[15]

The H-2 antibodies are complex. They may be formed against few or many components, and some components almost invariably act as strong antigens when present, for example, D and K. Others such as F, A, and H are usually effective only by hyperimmunization.

The H-2 antibodies, regardless of specificity, can exist in at least three forms, usually called saline, dextran, and blocking. Little has been done with the saline agglutinins in recent years. They are relatively unstable, are easily absorbed with red cells, and appear to depend for their detection on the physical state of the red-cell surface. Gorer[16, 17] showed ready agglutination of A-strain cells in saline, less consistent agglutination of C3H cells, and no agglutination of C57BL cells. White cells of all strains tested, including C57BL, are readily agglutinated in saline.[18] In the presence of a suitable dextran preparation and human serum, C57 red cells are agglutinated.[18] The dextran antibody appears more avid, absorption is more difficult with red cells, and the titers obtained are usually higher. Blocking antibodies are sometimes formed and are most frequently observed in sera produced in or against C3H, when they exhibit a prozone that may be very marked. If the nonagglutinating cells from the higher serum concentrations are washed and resuspended in a dextran-human serum mixture (but without additiona antibody), very strong agglutination occurs. It appears probable that antibody can exist in different physical states.

The X-type antibodies have been demonstrated mainly through thei ability to protect susceptible mice in passive-immunity experiments. They are not absorbed by normal tissues, are readily absorbed by the tumor against which they were produced, and have to date been studied mainly in lymphomas. Cross reaction has been observed in Gorer's laboratory between the X component of several, but not all, C57 tumors (Gorer, personal communication, 1957). Antibodies against X may be separated from the H-2 antibodies by absorption or by fractionation.[19] They are not cytotoxic in the presence of complement *in vitro* (Gorer, personal communication, 1957) and may act as "antiblastins" by inhibiting cell division.

Antibodies against the non-H-2 components probably resemble the H-2 antibodies, but are usually of low titer, sometimes so low as to be difficult to use in genetic experiments; it is to be expected from an analogy with the results of transplantation that their effect *in vivo* will be less strong, at least for the long-transplanted tumors, than the H-2 antibodies. Experiments in which protective serum from H-2 heterozygous immune mice was used showed only a very feeble effect.

Antigenic studies have been made on four C3H tumors, two carried in mice of the Heston subline (MC1M and K2S1) and two in the C3H Strong mice (6C3HED and C3HMT4). These differ in their transplantability and in their serologic behavior. MC1M was described as a rhabdomyosarcoma, but is now quite anaplastic; it kills a certain percentage of mice of many foreign strains even after prior immunization with blood. It appears to be a good H-2 antigen in that the antibody response to it is usually of high titer. Taken from the C3H strain, it absorbs antibody well *in vitro*. Like each of these tumors, it has certain individual characteristics. It tends to provoke an extreme cellular reaction when injected in moderate doses in C57BL,[20] and host cells may greatly outnumber the tumor cells; in this circumstance antibody may usually be detected in the animal's serum, but is frequently of rather low titer. If larger doses are injected, the cellular response is not observed, and agglutinins are detected less frequently. There is probably some disturbance of the normal antigen content of the cells, but no non-H-2 components have been detected to date.

As stated above, MC1M is strongly antigenic and will remove antibodies against H-2k efficiently upon first contact. If, however, fresh C3H liver, fresh MC1M, and lyophilized MC1M are mixed with a high-titer serum diluted one eighth for one hour, washed, and then used in a typical absorption experiment, the liver is found to be unsaturated and will continue to absorb antibody, the MC1M is much less avid, and the lyophilized suspension is almost ineffective, suggesting that the tumor cells, although more effective while still alive, are less capable of metabolizing or neutralizing antibody than had been supposed.[21] In an *in vitro* cytotoxicity test, the majority of cells were killed, but some appeared unharmed.

6C3HED is more strain specific, although recently it has killed a few mice of foreign strains. For a relatively specific tumor, it appears to carry unusually feeble antigens; the antibody response has a rather low titer, and it is often noticeably more efficient at absorbing antibodies directed against itself than those made against other C3H tumors. The relationships between its individual H-2 components appears normal, but there seem to be over-all fluctuations in antigenicity from time to time; recently it has been giving high titers in C57. It has one strong non-H-2 component that we have called alpha. This is produced consistently in the serum C57BL anti-6C3HED and reacts with 129 cells, although these are phenotypically similar to C57 in that both are H-2b. Antigen alpha is shared with at least 2 other strains, C and YBR, and is remarkable in that it is only feebly present on C3H/St tissues and red cells. A sample of serum produced after 4 injections of C3H/St liver and spleen into C57BL was a strong anti-K, but did not contain detectable anti-alpha. It is quite easy to remove the H-2 antibodies and leave anti-alpha intact by absorbing a high-titer serum with C3H/St liver. Anti-alpha will even survive 18 hours' passage through the whole C3H mouse in *in vivo* absorption, although the titer falls considerably. Passage through 129 or through a C3H mouse bearing 6C3HED lymphosarcoma removes anti-alpha completely.

K2S1 is a more recently derived mixed-cell sarcoma arising spontaneously in C3H/He. It is still highly specific and has only lately been grown successfully in C3H/St; it is at least a five-factor tumor in genetic tests with DBA/2. Antibodies to it are of interest for their extreme avidity. Two absorptions with C3H/He liver did not clear a serum having a quite modest titer, although it was subsequently found that a single absorption with K2S1 at the same serum dilution was sufficient.

C3HMT4 arose as a spontaneous mammary adenocarcinoma in C3H/St. At first highly strain specific, it has recently been grown in DBA/1. It has not been tested since this breach in specificity, but its strong H-2k antigenicity was comparable to that of any of the spontaneous mammary carcinomas tested. It is noteworthy that C3HMT4, Gorer's C3HMT1, and MC1M do not evoke anti-alpha in C57BL mice. C3HMT4 differs from the other tumors in that the breach of specificity observed in DBA/1 mice occurred after the second immunizing injection; the animals apparently rejected the first inoculum, but succumbed to massive tumors after the second. The tumor from these DBA/1's was transplantable to DBA/1, but not to C57. A similar situation has been encountered with the Dalton DBA/2 lymphoma, also grown in DBA/1. Here is was first observed that a few very young or very old animals succumbed to the first inoculation, but that young adult mice rejected the graft. After three or four injections, however, a number of them died from massive ascites.

When the experiment was repeated, serum was collected from animals with and without ascites, and the ascites fluid was also collected. There was little difference in the agglutinin level of the two groups, but the ascites cells from the pretreated animals could not absorb antibody, and there was free antibody in the ascites fluid. When these cells were injected into foreign strains they gave rise to an almost explosive initial ascites that later regressed. This same tumor has also shown a drastic change in transplantability and loss of antigenicity when passed through the chorioallantoic membrane of the chick before transmission through foreign strains;[22] regular passage of the regular tumor through DBA/1 for 1 year did not affect either the antigenicity or the transplantability for foreign strains.

The behavior in DBA/1 may be accounted for partly by the peculiarly feeble resistance offered by this strain. Noted by Snell and his colleagues[23] with several tumors, this has also been observed with skin by S. Grinnell in our laboratory.

Discussion

Evidence of incompatibility has been found in so many types of tumor, either through transplantation studies[2, 4] through the demonstration of localizing antibodies,[24, 25] or through the demonstration of neutralizing antibodies,[15, 26] that actual or potential defense mechanisms can be postulated against most types of tumor, certainly after transplantation, and even in the original host. The genetic composition other than the H-2 genotype of the host plays a great part in determining the behavior of a tumor graft.[23] This is most clearly seen in those tumors that can cross certain genetic barriers. Some of the variation appears due to the tumor itself, and some may be due to variation in the response of the host. The strain-to-strain variation can be capricious and unpredictable. For example, E.L.4, a C57BL leukemia, will grow in all C57BL H-2b sublines tested, but will grow exceptionally only in foreign strains; it has killed a number of F/St, generally considered a refractory strain, but under the same conditions did not kill DBA/1, usually the most susceptible strain we know. M. Potter (personal communication, 1956) found that P-288 from DBA/2 would not kill BALB/C, another H-2d strain, but we have found that it would kill DBA/1 mice.

Notwithstanding this type of inconsistency, H-2 factors overshadow all other genetic factors participating in the graft-host relationship, and it would be difficult to overemphasize this well-substantiated observation. The great majority of tumors are quite unable to grow when there is H-2 incompatibility. Part of the host response is due to the production of antibodies that, in the presence of complement, destroy the cells directly. That no host cellular participation is involved in at least certain instances can be shown by a refinement of the passive-immunity test, whereby lymphoma cells placed inside a millipore chamber die when antibody is injected into the animal. A strict compliance with H-2 compatibility holds for the bulk of tumors and forms the basis for the laws of transplantation.

Two broad categories of tumors are exceptional. One of these is also fairly easy to define, and includes such well-known tumors as the Ehrlich, Krebs, and a variant of TA-3. These usually violate all genetic barriers, and immunization against them is difficult. Some obviously possess antigenicity, for example, the hyperdiploid Ehrlich,[27, 28] but their antigenicity follows no known distribution and probably differs from clone to clone. These tumors are aneuploid and, in the chromosomal upheaval that has occurred, one ust suppose that the normal metabolic processes that lead to the formation f antigens have been disrupted, or else that a loss, imbalance, or mutation as involved the genes controlling antigenicity. The reason for their lack f specificity is that they do not elicit an adequate immune response rather han intrinsic resistance to antibody. Between these two groups is a third, etaining partial specificity but able to grow either in most members of a few trains (for example, A lymphoma No. 1) or in a variable proportion of a umber of strains (for example, MC1M).

Recently derived tumors may contain cells differing widely from one nother.[29, 30] Transplantation is selective, and the cells that grow rapidly nd do not stimulate an effective host response will predominate. A minority f other types of cells may persist and emerge when the environment becomes ore favorable to them. Increase in growth rate, more rapid killing of the ost, and simplification of genetic requirements usually develop progressively, ut not necessarily simultaneously, as the tumor is transplanted. These hanges may be the result of mutations, but there is evidence that the modi-ication in genetic requirements may result from a functional suppression of ntigens rather than a true mutational loss. As H-2-compatible backcross ice are unable to react against the non-H-2 antigens before being killed by the tumor, it is obvious that the antigens are no longer effective. That they still persist may be demonstrated in certain instances by triggering the immune mechanism of the host with a small amount of blood before injecting the tumor. The percentage of pretreated animals that develop tumors falls drastically.[31] In the same way, the first injection of a well-defined single-factor tumor such as A No. 2 into an incompatible mouse results in the formation of detectable H-2 antibodies only; the feeble antigenicity of the suppressed loci is, however, sufficient to trigger a more comprehensive response and, upon subsequent inoculations in the same host, other types of antibodies are also formed. This suppression is what one would expect if alternate metabolic pathways were available. The opening up of metabolic short cuts could be correlated with an increase in growth rate and should be considered an adaptation to parasitism.[32] The metabolites of the H-2 region would appear to be more essential to the normal functioning of the cell because they are consistently formed in relatively normal amounts until the cell establishes a completely new chromosomal balance in aneuploidy.[33]

From its originally complex state with a dependence upon the functioning of a large number of genes, the tumor arrives at a state where the only expressed histocompatibility factors are H-2's, although occult non-H-2 factors may still be carried. Some time after the tumor has arrived at the single-factor stage it may begin to kill in a certain percentage of a few foreign strains, DBA/1 being one of the most sensitive. The reasons for variation in the efficiency of the host defense have been little studied, but are pertinent and unexplained. From the work of Kaliss[34] and of Gorer[35] we know that certain concentrations of antibody may actually enhance tumor growth, whereas higher concentrations will inhibit. Anything that will delay or minimize antibody production may therefore be doubly beneficial to tumor growth. Many of the tumors of limited specificity may be feebly antigenic.

A-strain lymphoma No. 1 will grow in A and DBA/2. It is less efficient at absorbing anti-D or anti-K than its strain-specific companion A No. 2 (similarly X ray-induced in a litter mate), and gives a slower antibody response although, with repeated injections, the titers of antibodies against the two may be almost the same. L No. 1 appears to be even more deficient in the antigen K than it is in D, which may explain its greater compatibility for the DBA/2 strain than for, say, C3H.

Another known mechanism for genetic violation involves the coincidence of two different populations in a tumor. The variant of 6C3HED, adapted through a process of immunoselection to grow in DBA/2,[36] has two predominant cell types. The majority of the cells in the original tumor are near diploid; grown in DBA, near tetraploid. The tetraploid cell is a feeble antigen, the more antigenic diploid population persists in very low concentration, and probably does not offer an adequate antigenic stimulus to a mouse already in a state of precarious nutritional balance with the masses of tetraploid cells it bears. When the tumor is returned to C3H, diploid cells gradually outgrow the tetraploid, and a parallel increase in antigenicity is observed.

Dual populations may exist in other tumors, but the expansion of a less antigenic at the expense of a more antigenic population is probably not the only way in which the cytotoxic activity of a serum can be nullified. Surface modifications, either by combination with a blocking antibody or by adsorption of foreign protein, are possible. Even for sensitive cells, differences in lytic threshold for different tumor populations exist; E.L.4 is much more sensitive to antibody than, for example, 6C3HED, and lysis by antibody of MC1M appears to proceed more slowly than lysis of most other tumors. Antigenic loss is one known factor in loss of specificity, but each tumor is a composite mass of differing and varying cell types, and no single conclusion is likely to have a general application either to all tumors or even to all cells of a single tumor.

It is hoped that a study of the antigenic relationships and antigenic variability within tumors will be accelerated by the detection of some of the newer antigens and that it will be possible to place some of these factors in definite linkage groups.

Summary

A number of sublines and strains closely related to C3H/St have been typed serologically. With the exception of C/St, which is H-2q and has probably derived its H-2 antigens from its DBA parent, the strains and sublines are all H-2k.

Serologic differences, not at the H-2 region, exist between C3H/He and CHI on the one hand, and between C3H/St and CBA/St on the other. These differences have been partially analyzed and the probable distribution of three antigens tabulated. A fourth antigen was found in the tumor 6C3HED which, although shared by the red cells and tissues of three other strains, was only feebly present in C3H. Four C3H tumors were found to exhibit differences in their transplantability and in some serologic characters.

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ROLE OF CHROMOSOMES IN CANCEROGENESIS, AS STUDIED IN SERIAL TISSUE CULTURE OF MAMMALIAN CELLS*

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Introduction

Basing our views on observations of chromosomal behavior in cancers and following the classical views of Boveri, Winge, and others, we tend to regard the development of malignancy as a process of selection on the cellular level that favors cells with the acquired capacity of escaping the control of the host organism. The regular appearance of new karyotypes in cancer stemlines supports this view. In our opinion the significant feature in some kinds of cancerogenesis is the induction of genotypic variability. Any random genotype variation within a tissue may in time produce cells capable of neoplastic growth. Many facts indicate that the genotypic changes leading to this end are highly complex. Therefore, considerable experimentation with various genotypes will be necessary, and the transition from normal cells over precancerous to malignant cells will be floating. Natural selection within the cell population should automatically lead to cells with ever-greater independence of the host control. Because gross chromosomal disturbances and somatic segregation for chromatid and subchromatid changes will take place at mitosis, it is evident that any chromosomal instability will have an especially strong effect in tissues with high mitotic activity.

According to this view any mutagenic agent could be carcinogenic, provided the mutagenic action is extended over many mitotic cycles. Even one single exposure to X radiation would thus have a chance to bring about this effect if a specific type of mutation is induced in either the nucleus or the cytoplasm, that is, a mutation giving a permanent increase in mutability in all cells carrying this gene. This view is compatible with the chemical induction of cancer and perhaps will turn out to be so with the viral induction and transfer of the disease.

The change in environment connected with the explantation of mammalian cells from their normal position in the body into culture *in vitro* is indeed a drastic one. The fact that a high percentage of cells may survive such a change and may even start reproduction under the new conditions is good evidence of the considerable adaptability of mammalian cells. In many cases the adaptability is only temporary, and the tissue culture dies off after a few serial transfers. In other cases, however, a number of transfer generations with sluggish growth is succeeded by recovery and even increase of the cellular growth and multiplication rate. The tissue culture develops into a cell strain capable of indefinite survival *in vitro*. Instances are known when such cell strains have been carried serially for hundreds of transplant generations. Thus some strains of Gey (Gey *et al.*, 1954) have been carried *in vitro* for more than 15 years and, at the National Cancer Institute (Earle *et al.*, 1956), some clonal strains have likewise been carried for years. It may be safely assumed that such cells have undergone significant changes and deviate fundamentally from their original state. Actually, their development has been from an existence as integrated parts of a multicellular soma toward the habit of microorganisms living independently.

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One especially interesting change, demonstrated often to coincide with the establishment of the capacity for indeterminate growth *in vitro*, is the acquirement of malignant properties, made evident on inoculation of the cell strain *in vivo*. Realization of this fact, repeatedly encountered during recent years, makes mammalian tissue cultures a convenient and very welcome means for the study of premalignant stages, which are otherwise highly elusive of analysis.

The present investigation was planned as a study of the behavior of mitoses and chromosomes during serial transfer of tissue cultures, starting from the first mitosis occurring after explantation of the normal tissue and continuing until a change in the direction of malignancy was attained. We know that normal tissues *in situ* are characterized by a pronounced stability with respect to chromosomes. It is true that a certain incidence of endomitosis and probably other polyploidogenic mechanisms is typical of specific tissues, which accordingly will contain a fraction of cells with doubled or more highly multiplied chromosome number; but variations by parts of genomes, including intrachromosomal variations, are of little significance in normal tissues. We know also that malignancy is often accompanied by a loosening of chromosomal and mitotic constancy, variations both by single chromosomes and by parts of chromosomes being frequent. Little is known of the chromosome behavior between these two extreme conditions, that is, between the normal cell and the fully malignant cell, except for early responses of cells to carcinogenic hydrocarbons (reviewed by Biesele *et al.*, 1956). The present investigation was undertaken to help fill this gap.

The following alternative interpretations have been anticipated: (1) chromosomal instability in cancer tissue is merely a consequence of the malignant condition, and (2) cancer is the consequence of the earlier breakdown of chromosomal constancy. Our working hypothesis was that the change in environment involved in the growth *in vitro* would induce a genotypic variation. Thus becoming genetically heterogeneous, the cell population would form the material for selection leading to genetic adaptation of the cells for the new environment. It seemed reasonable that genetic adaptation to independent growth *in vitro* should often, although by no means always, condition the tissue in the direction of malignancy. Consequently, we believe this approach will have a bearing also on the chromosome situation when cancer develops *in situ.*

Material and Methods

Our choice of material was between human or murine cells for tissue culture. While the former undoubtedly would have greater general interest for the cancer problem, the murine cells have many practical advantages These include (1) the availability of inbred strains from which to start tissue cultures, (2) the possibility of challenging hosts of identical genotype with inocula of these tissue cultures, and (3) the structure of the mouse karyotype with its 40 chromosome arms all present as telocentric chromosomes and thus better suited for the detection of chromosomal changes than the karyotype of man, with its 92 arms joined into biarmed chromosomes of a great variety of types. Although we have experiments running also with human tissue cultures, the present report is based exclusively on murine material.

The tissue cultures were grown in a synthetic medium (Eagle, 1955) with added horse serum and, sometimes, chick embryo extract. For chromosome study, cells were grown on coverslips in Leighton tubes.

For the chromosome study, 2 different techniques were employed:

(1) The cells growing on the coverslips were fixed with alcohol-acetic acid and stained with the Feulgen nucleal reaction. This procedure has been used extensively for chemotherapeutic screening tests in the Sloan-Kettering Institute for Cancer Research. It is favorable for the detection of mitotic irregularities; our recordings of anaphase disturbances were made exclusively on such preparations.

(2) Cells grown on coverslips were treated for 1 hour before fixation with a colchicine concentration of about 10^{-6} mole/l. Immediately before fixation the cells were treated for a few minutes with hypotonic sodium citrate (generally 0.5 per cent solution for 3 to 10 min. gave good results, but concentration and time of treatment must be tested for each material). The coverslip with the cells was then placed on a droplet of acetic orcein (2 per cent orcein in 70 per cent acetic acid) on a siliconed slide. The staining was controlled under the microscope. When the chromosomes had taken stain the cells were squashed under constant microscopic control, the appearance of promising chromosome groups being followed during the squashing Tissue cultures are much more sensitive to squashing than solid tissues o ascites cells. By this method complete analysis of the chromosomes of sepa rate cells was feasible. This technique, which takes advantage of the tend ency of the cells to flatten out when grown on the coverslip, is a considerable improvement over the one earlier described (Levan, 1956a). The mos significant factor is the behavior of the staining solution, which must no form a precipitate with the citrate remaining in the cells. We do not know why some staining solutions are good, while others are useless. Adding citrate to the stain and then filtering seems to condition the stain favorably Usually, newly prepared staining solutions are unfavorable, while those stored for at least one month act better.

With these two techniques, we arrived at two different aspects: the forme echnique gave information about mitotic behavior, and the latter permitted n understanding of the karyotype responses to mitotic irregularities. 'ogether, they gave a consistent idea of the chromosomal events within the ells consequent to their explantation and serial passage *in vitro*.

The Mitotic Irregularities: Instruments of Cellular Adaptation

Note on the mitotic frequency. Cultures of normal mouse tissues are not asily workable for chromosome study. For the most part, their mitotic requency is lower than that of cultured tumor tissue, and their chromosomes ave a great tendency toward stickiness and are highly sensitive to handling. Jsually a lag period follows the explantation of a suspension of normal cells.)uring this period the cells settle on the coverslip, flatten out, and adjust hemselves to the glass surface, but do not enter mitosis. The lag period is specially evident after trypsinization, when it sometimes lasts longer than a reek. After mechanical mincing, on the other hand, there may never be ny mitosis-free period, solitary mitoses occurring from the start of the ulture. Normally, mitoses appear and increase in number after 1 or 2 days nd then again fade as the medium is exhausted. After refeeding the culture maximum of mitoses is reached, usually on the day after the refeeding. 'he maximum is especially pronounced if the feeding medium contains 0 per cent chick embryo extract. Occasionally one may obtain mitotic ndices of 4 to 5 per cent, which are very high for early passages of normal issue. The most favorable day for chromosome fixation is the day after eeding.

TABLE 1 summarizes mitotic indices for some of the freshly set up cultures f skin and brain tissue. In every case a mitotic maximum is reached 1 or days after feeding of the cultures.

TABLE 1
MITOTIC INDEX OF CULTURED CELLS OF NORMAL TISSUES

Tissue	Treatment	Exp. No.	Days after start of experiment								
			1	2	3	4		5	6	7	8
kin.......	Trypsin 3 hr.	36	0.4	1.0	1.7	0.6	Feeding of cultures	2.1 4.5*	0.3	0.2	0.1
rain......	No trypsin	110	—	1.5	1.5	1.9		2.9	3.3	2.4	1.6
rain......	Trypsin 1.5 hr.	108	0.8	0.8	1.5	1.1		3.5	1.3	1.1	—
rain......	Trypsin 3 hr.	111	—	0.3	0.6	0.2		2.6	2.5	1.1	0.5
rain......	No trypsin	208	0.0	1.0	1.2	1.2		2.5	2.1	3.0	—
rain......	Trypsin 3 hr.	207	0.3	0.0	1.6	1.4		4.4	2.2	2.4	—

* Twenty per cent chick embryo extract.

On the successful passage of the first generation, a second generation is ormed; this usually grows more slowly and has a lower mitotic index than he first generation. A decidedly sluggish growth is usually characteristic of a number of subsequent transplant generations, and many of the cultures die off during this period. We have followed brain tissue, liver, and spleen for 2 to 3 transplant generations, but only in the case of skin tissue have we succeeded in getting past the period of sluggish growth. As described in greater detail below, one skin culture (experiment 76) has been followed through 36 generations. From approximately generation 15 this culture started growing rapidly, and its mitotic frequency increased to the leve typical of tumor tissue cultures. In fact, its behavior suddenly started to resemble that of the Foley strain of mouse sarcoma 180, which we also carry in culture. Fortunately, its karyotype is entirely different from that of the Foley strain, so suspicions of contamination with this tumor could be ruled out. Others of our skin cultures are in generation 10 at the time of writing and still grow very hesitantly.

The frequency of irregular mitoses. We were surprised to find, in every culture examined, a number of cells showing chromosomal irregularities. In order to arrive at an estimate of the frequency of these irregularities we studied all anaphases and telophases in the slides of certain experiments. Only slides prepared according to the Feulgen method were used for this purpose. The cells in this case had undergone no vital pretreatment before fixation.

In the following, cells with abnormal anaphases and telophases are recorded as percentages of the total number of cells at these stages (%AA). As is brought out in greater detail in the next section, the %AA involves a generalization, combining under one heading every chromosome irregularity apparent toward the end of the mitotic cycle. Thus, the disturbances, here called AA, range from nondisjunction and vagabonding of entire chromosomes to the appearance of acentric and dicentric chromosomes and chromatids. They comprise both numerical and structural irregularities. Since our aim was to estimate the significance of mitotic irregularities in the creation of genotypic variation in the tissue cultures, we tried to include in the %AA all disturbances that may contribute to this end.

Although our %AA thus takes into account all visible anaphasic and telophasic abnormalities, it will certainly always give a minimum representation of the real genotypic variation in the tissue culture. Only specifi classes of structural rearrangement will be visible at anaphase. Also, since many of the recorded cases involve morphologically highly insignifican changes, which are really on the border of visibility, the occurrence of many similar changes may be assumed to elude observation. For evolutionar trends, the morphologically most insignificant changes must be considere the most promising ones.

In the present section a few experiments are analyzed as to their %AA TABLE 2 gives these data for experiment 36, the third generation of an embry onic skin culture after trypsinization. It appears that, of 872 cells, 11 pe cent were abnormal. In the separate cultures the %AA varied from 4. to 18.9.

In embryonic skin a considerable fraction of the nuclei is ordinarily o larger size, and a corresponding proportion of the mitoses accordingly i etraploid or octoploid. This was also the case in the explanted skin tissue. An attempt was made in some of the cultures to divide the recorded %AA nto diploid and tetraploid cells, respectively. As seen from the last column of TABLE 2, the %AA was considerably higher in the tetraploid cells.

The result of similar analyses of some experiments with embryonic brain cells is recorded in TABLE 3. Experiments 110 and 111 of this table were ollowed through 2 generations. Both at the original explantation and at the transfer to generation 2, the cells of experiment 110 were handled without rypsin, while those of experiment 111 were trypsinized both times. The able indicates one striking fact: experiment 110, with its mechanically minced tissue, has a persistently lower frequency of AA. The %AA is 1 to 5 in this experiment, while it is about three times higher (8 to 18 per cent) in experiment 111, set up with trypsinization. These results suggest that the trypsin treatment may involve a potent mutagenic action. It should be mentioned, however, that the conditions of experiments 110 and 111 differed in respects other than the use of trypsin. Experiment 110 was planned as a control and was treated as mildly as possible. Consequently, the cells were planted directly after the mincing, while the cells of experiments 108 and 111 were digested with the trypsin under stirring for 1.5 and 3 hours, respectively.

TABLE 2
%AA OF THE THIRD TRANSPLANT GENERATION OF EXPERIMENT 36
(EMBRYONIC SKIN CELLS)

Day after start of exp.	Culture number	%AA	Number of cells examined
2	5	9.8	82
	7	11.3	106
	8	8.0	100
3	9	12.5	56
	10	7.8	102
	12	12.2	41
5	17	7.5	106
	19	18.9	160
	20	4.6	86
6	23	12.1	33
Total		10.9	872
Total* 2x		9.1	547
Total* 4x		24.4	78

* Cultures Nos. 7, 8, and 12 excluded.

For testing whether the difference would persist even if the control cells were stirred with normal medium for the same length of time as the cells of the trypsin series were stirred with the trypsin solution, experiments 207 and 208 were planned. Embryonic brain tissue was homogenized, and the pool of cells thus obtained was divided into 2 lots, one of which was digested for 3 hours with trypsin; the other was left for the same time in medium without trypsin. Both were stirred magnetically for the entire digestion time. The result of these experiments is seen in TABLE 4. It is clearl indicated that the trypsinized cultures, even in these experiments, had higher %AA than the nontrypsinized cultures.

TABLE 5 is a summary of our data on this remarkable difference in %AA between trypsin and no trypsin. Although a certain overlapping of th

TABLE 3

%AA OF THE FIRST GENERATION OF EXPERIMENT 108, AND OF THE FIRST AND SECON GENERATIONS OF EXPERIMENTS 110 AND 111 (EMBRYONIC BRAIN CELLS)

Generation	Days after feeding	Culture number	Experiment 110, no trypsin	Experiment 108, trypsin 1.5 hr.	Experiment 111, trypsin 3 hr.
1	1	7	3.0	12.0	13.0
	1	8	5.0	14.0	15.0
	2	9	1.0	21.0	16.0
	2	10	2.0	12.0	15.0
Average			2.8	14.8	14.8
2	(Before feeding)	15	3.3	—	8.0
	(Before feeding)	16	4.2	—	16.0
	(Before feeding)	17	—	—	18.0
	(Before feeding)	18	2.4	—	16.0
	1	19	—	—	13.0
	1	20	—	—	13.0
Average			3.3	—	14.0
Total average			3.0	14.8	14.3
Number of cells examined			496	300	1000

TABLE 4

%AA OF THE FIRST GENERATION OF EXPERIMENTS 207 AND 208 (EMBRYONIC BRAIN CELLS)

Days after feeding	Culture number	Experiment 208, no trypsin	Experiment 207, trypsin 3 hr.
(Before feeding)	5	4.2	10.9
(Before feeding)	6	4.2	14.3
(Before feeding)	7	6.9	23.9
(Before feeding)	8	8.6	8.6
1	9	2.2	16.0
1	10	3.8	8.6
2	11	2.0	13.0
2	12	4.0	10.5
3	13	3.2	9.9
3	14	8.0	10.0
Average		4.7	12.6
Number of cells examined		670	762

TABLE 5

SUMMARY OF %AA IN NONTRYPSINIZED AND TRYPSINIZED CULTURES OF EXPERIMENTS 36, 108, 110, 111, 114, 115, 207, AND 208

Treatment	%AA					Total samples	Average %AA
	0–5	5–10	10–15	15–20	20–25		
No trypsin........	14	7	—	—	—	21	4.4
Trypsin...........	1	8	15	8	2	34	12.8

distribution series is found in the region of 5 to 10% AA, without doubt our results show a greater tendency to AA after trypsin treatment than after manual mincing. Since trypsinization, performed in one way or another, is a standard procedure in some modern tissue culture techniques, both in setting up original explants and in making transfers, our results are highly suggestive. If trypsinization is in reality equal to a powerful mutagenic treatment, it will increase the genotypic variation of the culture. This means a richer material for selection and improved chances for survival of he culture. Trypsinization, now so popular in tissue culture work, may have a twofold action: (1) in helping to establish new cell strains from tissue cultures, and (2) in enhancing the departure from the cell type of the normal tissue of origin.

On the suggestion of W. Nowinski, we made a preliminary experiment substituting hyaluronidase for trypsin as a means of obtaining separate cells n suspension. We used a pharmacological preparation called Alidase*

TABLE 6

%AA OF THE FIRST GENERATION OF EXPERIMENTS 114 AND 115 (EMBRYONIC BRAIN CELLS)

Days after last feeding	Culture number	Experiment 114, no hyaluronidase	Experiment 115, hyaluronidase
3	5	5.8	6.5
3	6	8.2	2.9
1	7	4.4	0.9
1	8	5.3	6.3
Average %AA.................		5.9	4.2
Total number of cells examined...		191	296

n a concentration of 25 viscosity units/cc. As seen in TABLE 6, no difference n %AA is indicated between the control and the cultures treated with hyaluronidase. The values for both series, however, seem to be somewhat higher than in the controls of preceding experiments. We do not know the elative efficiencies of our concentration of hyaluronidase and our concentration of trypsin in separating cells. This question will be studied with a tissue such as skin, in which the cells are joined into a tougher texture tha are brain cells. The results of the present experiment are given solely as instances of AA frequencies.

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* G. D. Searle & Co., Chicago, Ill.

In summing up this section, it may be stated that, even with the mildest conditions of preparation, newly set up tissue cultures will exhibit a con- siderable frequency of mitotic disturbances. Evidently there is an unavoid- able "background" mutation rate, which may be raised by changing the environment, as, for instance, by exposing the cells to trypsin.

TABLE 7

OCCURRENCE OF DIFFERENT TYPES OF AA, AS OBSERVED IN THE FIRST THREE GENERATIONS OF TISSUE CULTURES

Abnormal anaphases	Trypsin	No trypsin	Total numbers	Percentages
With acentrics alone				
One acentric	178	20	198	43.6
Two paired, of equal length	46	8	54	11.9
Two, nonpaired, or more	64	14	78	17.2
Total AA with acentrics	288	42	330	72.7
With bridges				
One bridge	36	16	52	11.5
One bridge and one acentric	20	4	24	5.3
One pseudochiasma	11	3	14	3.1
Two or more bridges, with acentrics	29	5	34	7.5
Total AA with bridges	96	28	124	27.4
Grand totals	384	70	454	100.0

The morphology of irregular mitoses. Instances of the chromosomal dis turbances discussed in the preceding section are pictured in FIGURES 1 and 2 All cases recorded are summarized in TABLE 7, with an attempt at crud classification. The changes range from very small, hardly visible acentri fragments which, genetically, should mean small deletions, to highly drasti changes in which most of the chromosome complement is engaged in struc tural rearrangements.

The most common change, representing 44 per cent of all disturbances, i the presence of an acentric fragment between the anaphase groups, or a micro nucleus outside the telophase nuclei. The excluded body may vary in siz from extremely small to huge, in some cases reaching the combined lengt of 2 chromosomes (FIGURES 1*a*–1*e*). In this group, cases of 1 vagrant chromo some also have been included; these arise from spindle failure, not fro structural change. A specific type of acentric fragment is the group wit paired symmetrical fragments (FIGURES 1*w*, 1*ii*, 1*rr*, and 2*n*) or 1 fragmen folded together at the middle (FIGURE 1*m*). This type indicates an earl break before the chromosomes have begun reacting as double. Exceptional ring-shaped acentric fragments are observed (FIGURE 1*s*). Cells with 2 or more fragments are common (FIGURES 1*h*–1*j*). Some of these cases also may include vagrant whole chromosomes. Cells with chromosome bodies outside the developing daughter nuclei represent 73 per cent of all irregularities recorded.

The remaining 27 per cent are cells with bridge formations. One bridge without fragment (FIGURES 1*bb*–1*ee*) or one with an acentric fragment (FIGURES 1*z*, 2*a*) make up 17 per cent of the cases. Approximately 7.5 per cent had 2 or more bridges and usually, in addition, several fragments. In the latter group are included some highly drastic changes that are occasionally seen (FIGURES 1*oo*, 1*rr*, 2*f*–2*l*). A characteristic case among bridge formations is that of crisscross bridges, indicating presplit interchromosomal translocations (FIGURE 1*jj*). Typical pseudochiasmata were repeatedly observed (FIGURES 1*u*, 1*v*, 2*d*, 2*e*). They are comparatively rare, however, representing only 3 per cent of all disturbances.

In addition to the purely structural disturbances, there is (as indicated above) also a variation with whole, unchanged chromosomes, that is, a numerical variation. Apart from vagrant chromosomes, classified in TABLE 7 together with the acentrics, among the cells examined, about 20 with serious spindle disturbances, such as multipolarity, were recorded.

Considered in its entirety, evidence collected from the first and second transplant generations of mouse cells in tissue culture indicates that all kinds of mutation processes are at work, resulting in numerical and structural chromosome changes. It has been shown that these processes may affect even the first mitoses after the isolation of the tissue from the host body.

Insofar as we know, the normal tissue at the moment of explantation consists of cells with the same chromosome complement (with possibly a number of doubled cells present) or, genetically speaking, belonging to the same (or the autotetraploid) biotype. The mutation processes, starting in the tissue immediately after its explantation, theoretically may lead to one of the alternative conditions: (1) *status quo* may be maintained in the tissue because of a rigorous antiselection against any changed genotype, or (2) some of the altered genotypes may survive, the tissue changing into a population composed of different biotypes. If the first alternative were true, cancer might never have developed. On the contrary, changed cells may survive and new deviating karyotypes may appear in the tissue culture soon after its explantation.

The genetic diversity thus induced in the tissue culture will increase steadily as new aberrations are continuously released by the mitotic mutation process. This situation prevails either until the tissue culture fades out or until some truly superior cells happen to appear. These genotypes will then outgrow all less viable types, and the tissue culture will again enter a period of relative homogeneity. This homogeneity will be of a nature other than that of the original normal tissue, resulting from the superiority of a certain chromosomal constellation, a superiority always challenged, always to be defended. The relatively static conditions of the normal tissue have been succeeded by the dynamic equilibria of cancer tissue.

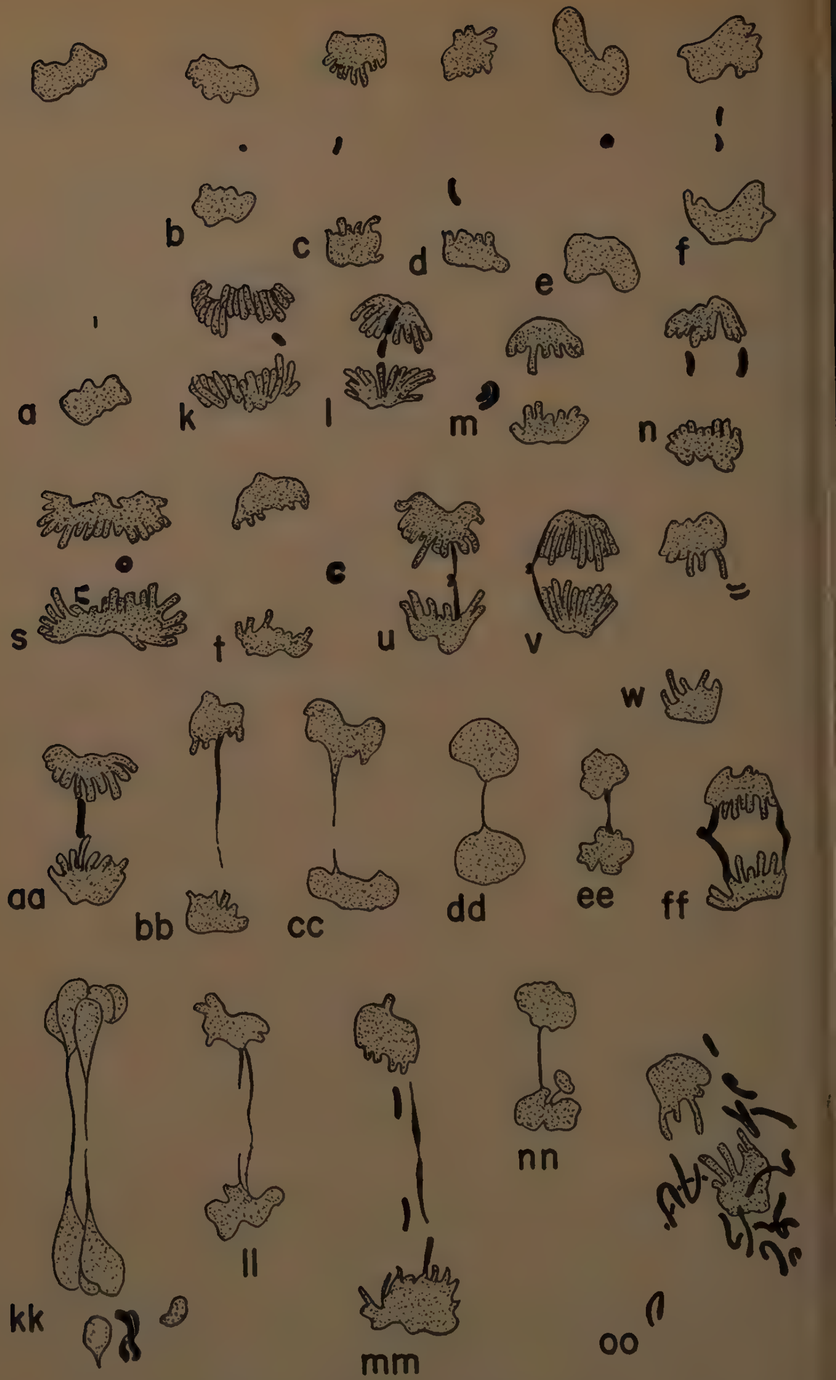
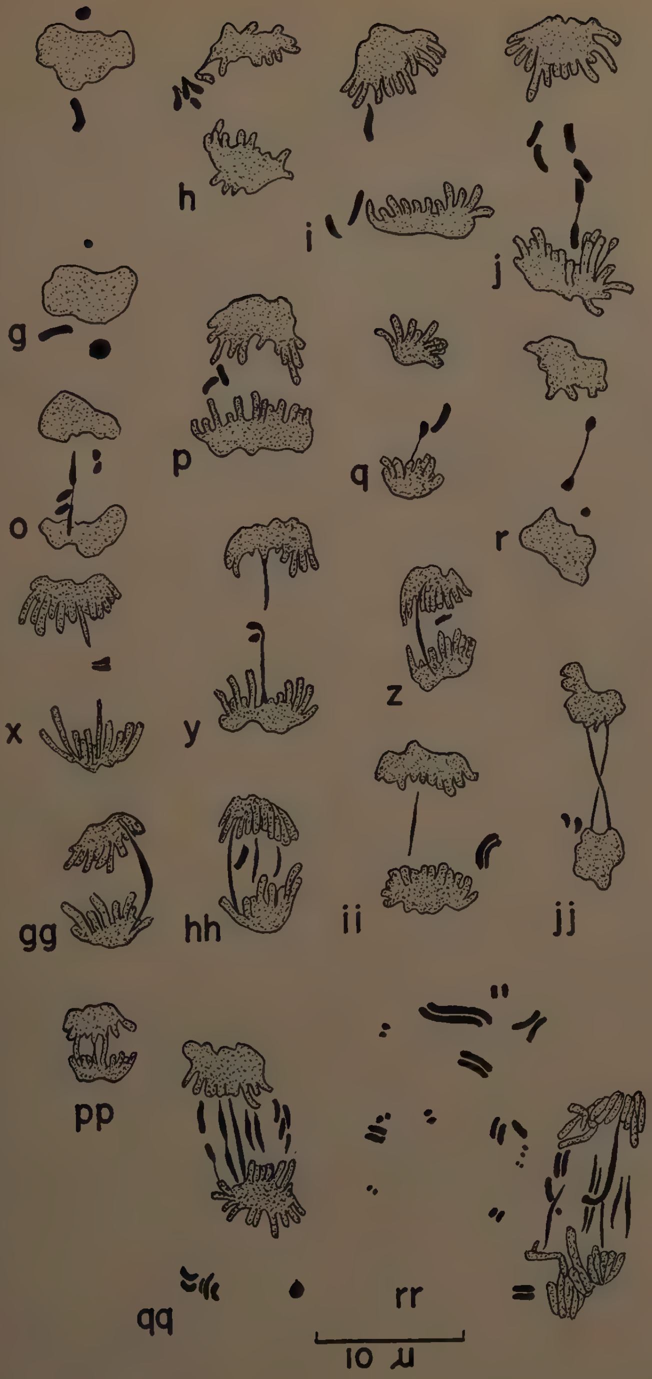


FIGURE 1. Forty-four cases of abnormal postmetaphases of first-generation tissu cultures of embryonic mouse brain, fixed 1 or 2 days after refeeding. All, except *a*, *s*, an *jj*, are from 2 comparative experiments: 110 (no trypsin) and 111 (trypsin). Eleven o the cases (*e*, *f*, *i*, *l*, *n*, *q*, *v*, *ee*, *gg*, *ii*, *pp*) are all AA's found among 400 postmetaphase studied in experiment 110 (%AA = 2.8), while 28 of the remaining 30 cases are all AA'



found among 200 postmetaphases of experiment 111 (%AA = 14.0). The disturbances include acentrics (*a* to *n*), bridges with or without acentrics (*aa* to *nn*), an acentric ring (*s*), crisscross bridges (*jj*), pseudochiasmata (*o* and *y*), and extreme rearrangements (*oo*, *qq*, *rr*). Carnoy, fixation, Feulgen stain. ×1900.

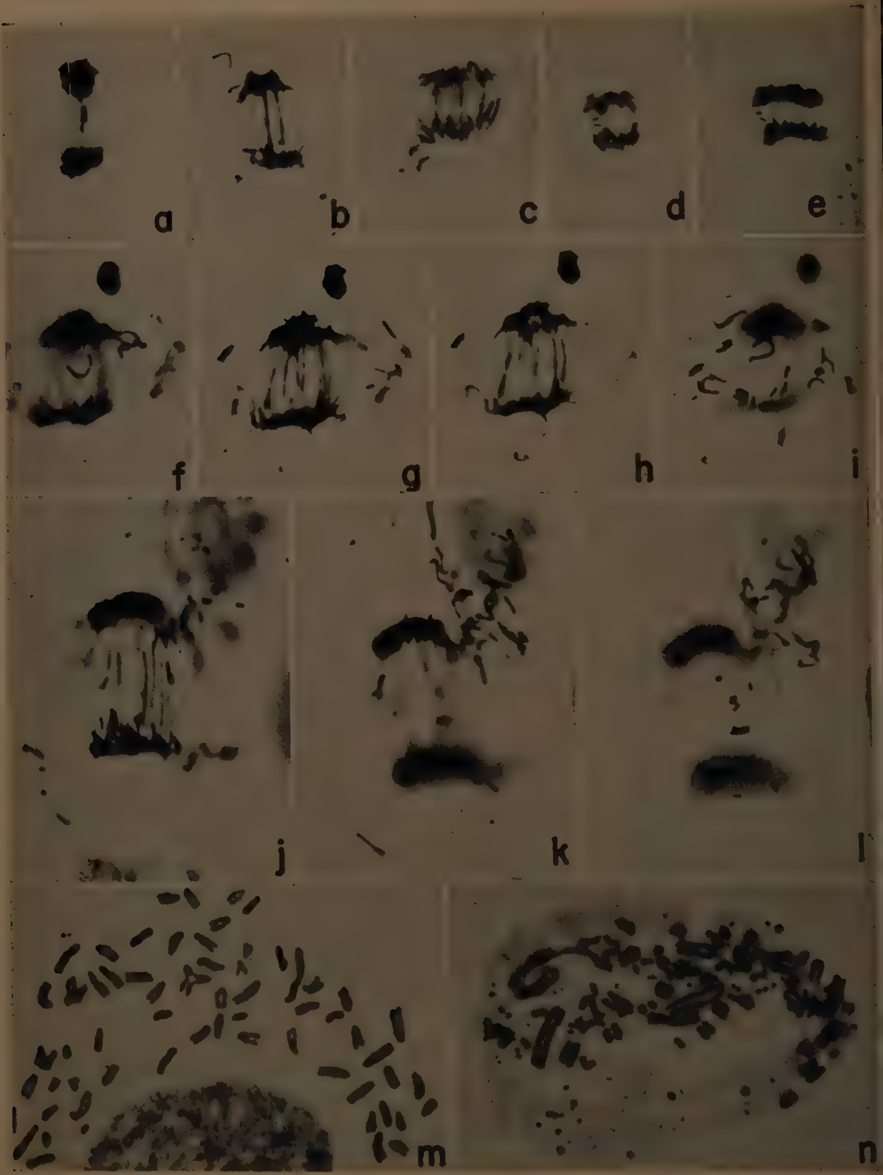


FIGURE 2. (*a–l*) Instances of various anaphase disturbances; (*m* and *n*) 2 c-metaphases; (*a–c*, *f–l*) bridges and acentrics; (*f–i* and *j–l*) 2 cells at different foci; (*d–e*) pseudochiasmata; (*m*) 1 triradiate; (*n*) extreme chromosome rearrangements. (*a–l*) Carnoy fixation, Feulgen stain; (*m* and *n*) acetic orcein. ×1000. FIGURE 2*n* is reproduced by permission from J. J. Biesele, *Mitotic Poisons and the Cancer Problem*, Elsevier Publ. Co., 1958, page 93.

The New Karyotypes: Steps in Cellular Adaptation

If some of the mitotic disturbances studied in the preceding section lead to viable cells capable of reproduction, we should expect to find, from the very beginning of the *in vitro* culture, metaphases with deviating chromosome numbers and chromosome types. With the aid of the acetic orcein technique described above, we were able to obtain idiogram analyses of entire cells.

Embryonic skin tissue. Tissue cultures of embryonic skin proved very favorable for chromosome examination. The average chromosome shape, which varies from tissue to tissue, is delicate and slender, chromosome details being very clear. The early generations were studied in four experiments. Thus far, later generations have been accessible in only one experiment.

TABLE 8
DIPLOIDY AND POLYPLOIDY IN SKIN CULTURES

Experiment number	Generation number	Percentage				Number of cells examined
		±2x	±4x	±8x	±16x	
80	1	65	31	4	0	110
81	1	37	52	11	0	52
90	1	53	26	21	0	19
	4	21	79	0	0	14
91	1	56	33	9	0	96
	2	29	71	0	0	7
	4	24	58	18	0	17
76	9	29	58	11	2	28
	11	8	74	17	1	72
	16	0	91	5	4	43
	17	0	87	13	0	23
	18	0	79	11	10	19
	19	0	90	7	3	29

The presence of a high frequency of approximately tetraploid cells and also of a fair number of cells with octoploid and still higher chromosome numbers is characteristic of the explant generation of the skin cultures. This is a direct consequence of the condition of skin tissue *in situ*, for it contains a considerable proportion of giant nuclei. TABLE 8 and FIGURE 3 show the frequency of metaphases on the different ploidy steps in the culture examined. In three cases, generation 1 contains a majority of diploid mitoses, but in one case the polyploid mitoses are already more frequent at this stage. In the samples of generation 4 examined, there is a clear tendency of the polyploid fraction of mitoses to increase in number. This tendency is even more clearly defined in generations 9 and 11 of experiment 76, the only one carried that long. By the time this latter experiment could be examined again (that is, in generation 16), the diploid fraction had totally disappeared.

This change occurred during the period when this culture had begun growing with greater speed. Samples from later generations have never contained any mitoses from the diploid range of chromosome number.

However, the five cultures of skin cells examined undoubtedly follow a certain pattern in their development during serial growth *in vitro*. The diploid region loses in importance for each transplant generation in favor of the tetraploid region, which eventually comes to predominate completely. When a vigorously growing cell line is finally formed, its stemline is selected from the tetraploid region. This development, with respect to the disappearance of diploidy, is represented in FIGURE 3. It should be observed that this pattern is typical of the present material, which thus far is fairly meager, and other patterns may quite possibly appear on other occasions.

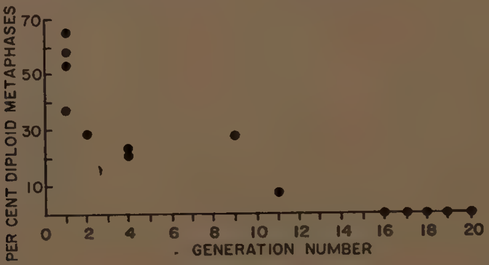


FIGURE 3. Disappearance of diploidy from skin cultures.

It was possible to go somewhat beyond the general pattern now described, because many metaphases were good enough to allow exact chromosome counts. In TABLE 9 exact counts have been compiled for the different stages of serial transplantation of the skin cultures: generations 1 to 4 represent the chromosome numbers soon after the explantation; generations 9 to 11, the period of sluggish growth; and the last samples, from generation 16 on, are from the period after the shift into a cell line had occurred.

It is an interesting fact that during early generations euploid numbers predominate, at generations 1 to 4 the diploid number, and at generations 9 to 11 the tetraploid number. After the shift into a cell line, euploid numbers are respresented no more often than adjoining numbers, and the role of euploidy as a carrier of superior viability has expired, certain aneuploid numbers now being equally viable or superior. Aneuploid numbers are present from the beginning. In the diploid region we found $2x - 1$ as well as $2x + 1$ and $2x + 2$, and in the tetraploid region there is a clear tendency to variation downward from the tetraploid number. It is from the hypotetraploid region that the new stemline develops later. Between generations 16 and 23 the chromosome number range is very wide (53 to 95). There is a tendency to bimodality, one mode being between 60 and 70, the other between 70 and 80. The next chromosome counts available were from generations 35 and 36. Here the distribution has narrowed down considerably, a very pronounced mode, comprising 70 per cent of all cells, being present in the region from 74 to 77. It was to be expected, of course, as

TABLE 9
EXACT CHROMOSOME NUMBER COUNTS IN SKIN CULTURES

Generations	Chromosome number																																				Total cells examined
	39	40	41	42	47	53	58	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	88	93	95	
1–4	6	87	4	1	1	—	—	—	—	—	—	—	—	1	—	—	1	—	—	1	1	2	3	2	1	6	3	41	3	1	1	—	—	—	—	—	166
9–11	—	12	2	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	2	—	4	1	3	3	1	3	—	44	—	—	—	2	—	—	—	—	79
16–17	—	—	—	—	—	1	1	1	2	—	3	4	5	3	3	4	3	1	3	2	5	3	4	5	—	1	—	2	—	1	—	—	—	1	1	—	59
18–19	—	—	—	—	—	—	1	—	—	—	—	1	—	2	3	1	3	—	1	4	2	1	8	5	2	3	1	1	—	—	—	—	1	—	—	1	41
20–21	—	—	—	—	—	—	—	—	—	1	2	1	—	2	1	1	—	—	1	1	4	5	2	4	2	3	—	1	—	—	—	—	—	—	—	—	31
23	—	—	—	—	—	—	1	—	—	2	—	3	4	8	10	11	7	6	7	4	9	9	4	8	4	1	1	—	—	1	—	—	—	—	—	—	100
35–36	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	3	6	7	21	25	14	10	4	4	1	—	1	1	1	—	—	—	—	100
After *in vivo* passage.......	—	—	—	—	—	—	—	3	1	1	2	5	4	4	3	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	25

favorable mutations accumulated in a certain chromosome number, that the numerical variation would become more concentrated, as usually happens with well-established, transferable cell lines and tumors.

The tissue cultures of experiment 76 were tested for *in vivo* transplantability from generations 18, 22, 23, 26, and 33. These experiments will be described in greater detail elsewhere; for the present we need mention only that the first positive result was with generation 22, from which a tumor was grown and later diagnosed by Fred Stewart and Fred Shipkey of the Pathology Department of Memorial Center for Cancer and Allied Diseases, New York, N. Y., as a spindle cell sarcoma (FIGURE 4). Later, other samples from the cultures of experiment 76 gave tumor growth. The tumors were transplantable from animal to animal and have been re-explanted *in vitro*.

The last line of TABLE 9 reports 25 counts from a tissue culture explanted from a second-generation *in vivo* growth, started *in vivo* from generation 22 *in vitro*. It is of very great interest that the chromosome numbers of this culture show a narrow distribution in the sixties, being thus quite distinct from the continuous *in vitro* cultures. It seems that the existence *in vivo* has selected the lower of the two chromosome number modes mentioned above, while the continued growth *in vitro* has evidently favored the higher mode.

The appearance of the chromosomes in some cells from the skin cultures may be seen in FIGURE 5: *a*, *b*, and *c* are instances of metaphase plates from the diploid region with 39, 41, and 40 chromosomes, respectively; *h* and *k* are hypotetraploid plates with 79 and 76 chromosomes; while *i* is a hypo-octoploid plate with 153 chromosomes. FIGURES 6*a*, 6*d*, and 6*f* are photomicrographs of mitoses in skin cells.

Throughout this study it was observed that the regularity found in mouse tumors (Levan, 1956b), namely, the greater tendency toward structural rearrangements in the tetraploid region than in the diploid region, appeared also in the present material. While the chromosomes of diploid cells can usually be arranged into pairs of homologues without difficulty, tetraploid plates very clearly do not consist solely of quartets of homologous chromosomes. While this would be difficult to demonstrate for chromosomes of intermediate length, it is easily seen for the extreme chromosome types, that is, the largest and the smallest ones. The diploid plate of FIGURE 5*c*, for instance, may easily be arranged into 20 pairs, but the hypotetraploid plate of FIGURE 5*h*, on the other hand, will refute any assumptions of autotetraploidy. FIGURE 5*e* shows a higher magnification of its 2 largest and 3 smallest chromosomes. They both fall outside the variation range of the diploid idiogram, and they are not members of 2 quartets of large or of small chromosomes, respectively. All 5 of them are individual types of 5 different sizes, as is especially clear in the case of the small ones. This is morphologic evidence that not only numerical but also structural changes take part in the formation of new karyotypes appearing in tissue cultures. Similar cases are FIGURE 5*k*, which has a single, very small chromosome, and the hypo-octoploid plate of FIGURE 5*i*, in which the smallest chromosomes do not form an octet of identical type. The condensed idiograms of FIGURES 5*d* and 5*f*

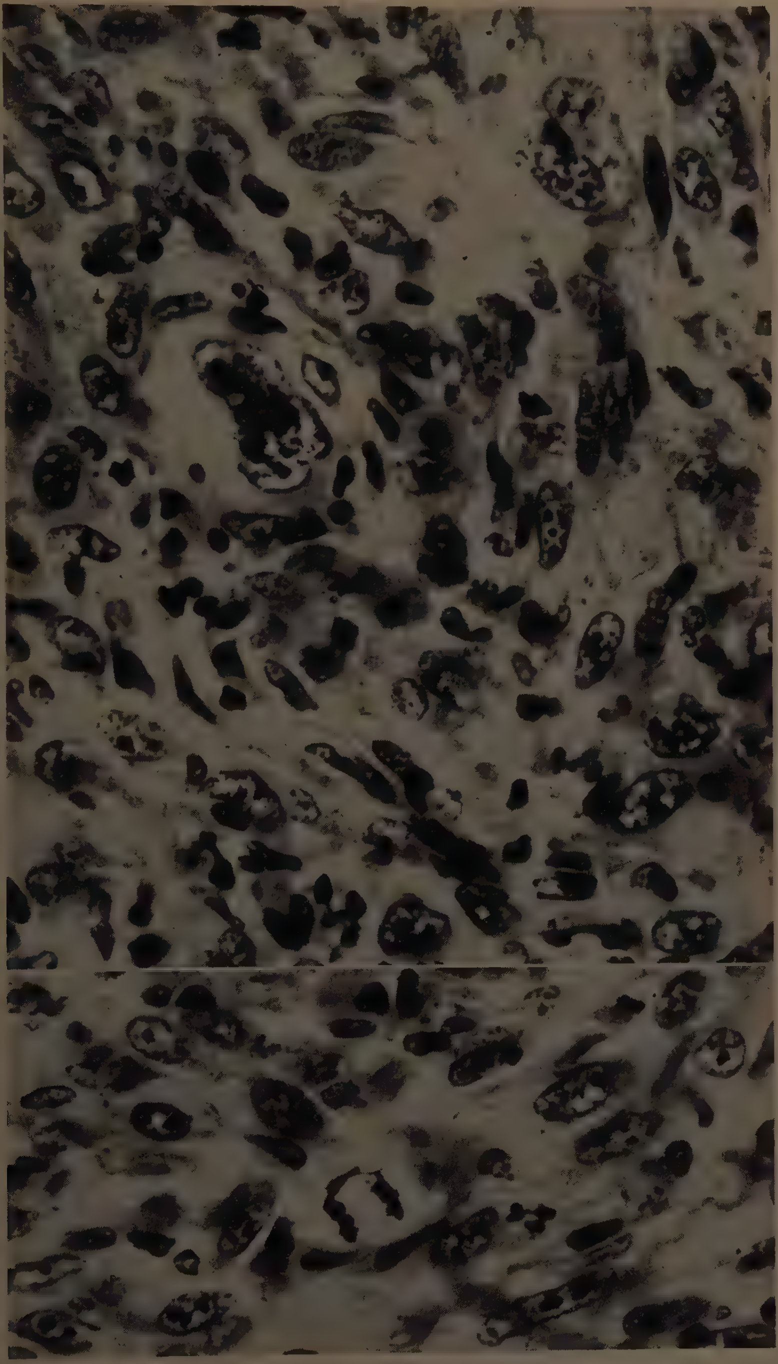


FIGURE 4. Spindle cell sarcoma developed from embryonic skin culture. Bouin fixation, hematoxylin-eosin stain. ×740.

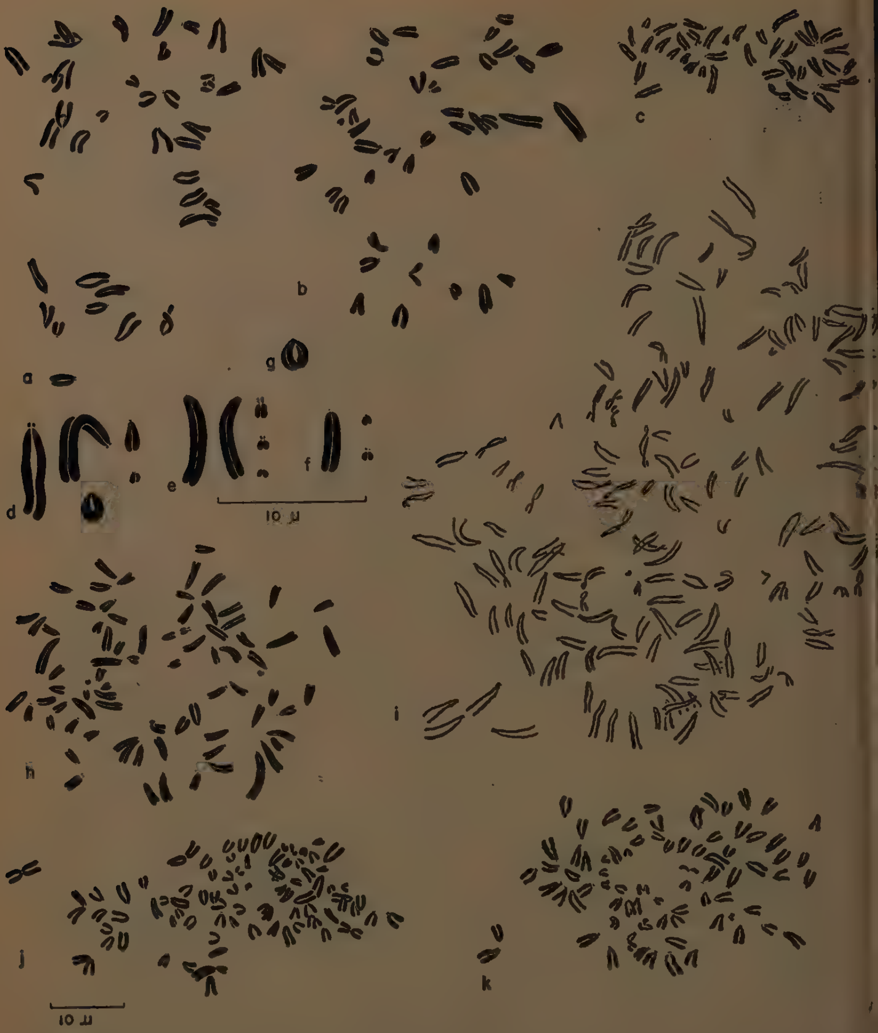


FIGURE 5. Metaphases from skin cultures of experiments 76 and 90. The following transplant generations are represented: 1 (*a*, *b*, *d*, *g*), 4 (*e*, *f*, *h*), 9 (*c*, *k*), 16 (*j*), and 19 (*i*). (*a–c*) Near-diploid numbers, 39, 41, and 40; (*h*, *j*, *k*) near-tetraploid numbers, 79, 88 arms, 76; (*i*) near-octoploid number, 153; (*d–g*) characteristic chromosomes of certain near-tetraploid cells, the largest and smallest chromosomes, ring chromosomes. Acetic orcein stain. (*d–g*) ×1900; the rest ×950.

show the same. In 5*d*, as in 5*g*, a typical ring chromosome is present, probably a transient sign of the structural rebuilding at work.

Both the structural and the numerical variations are more limited at the diploid level. It is of interest that chromosome plates with 39 chromosomes were found. Usually counts below 40 were disregarded, since chromosomes are easily lost in the squashing, but some very evident cases convinced us

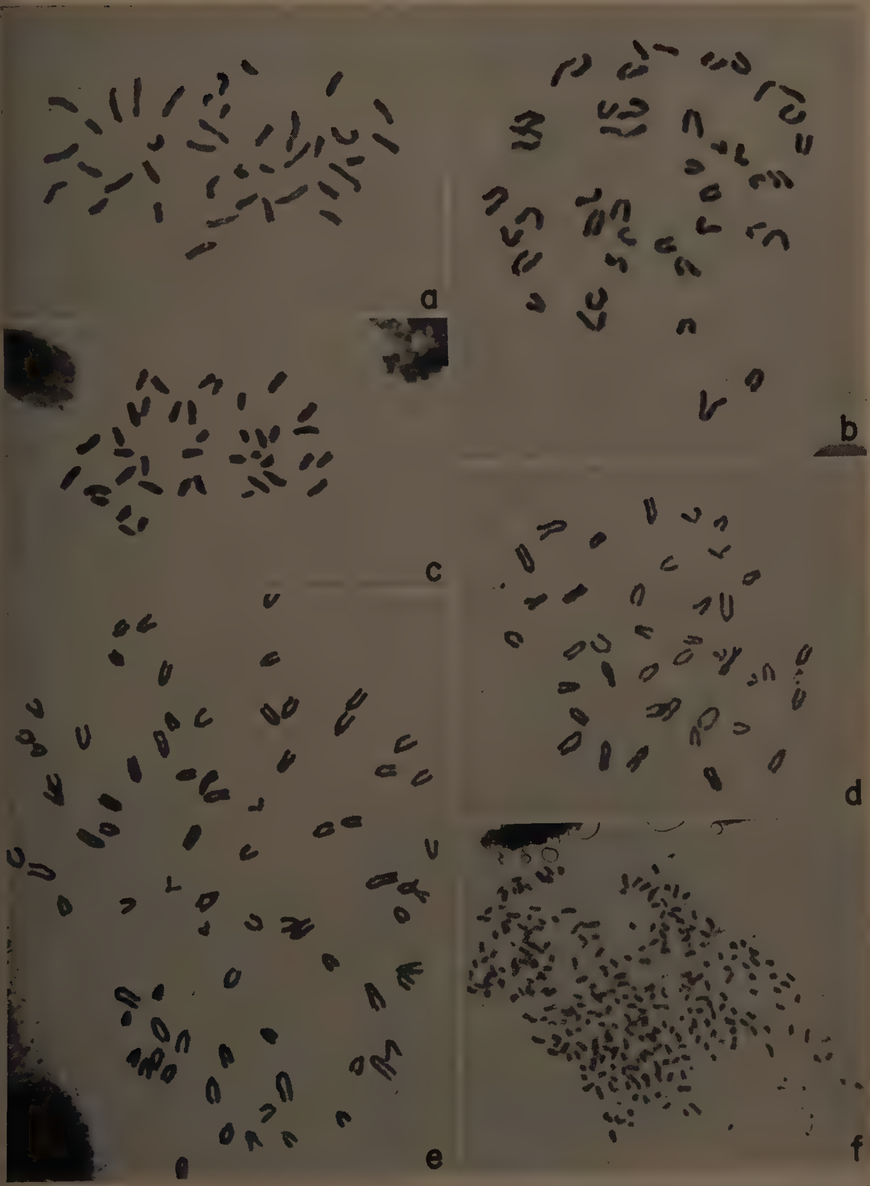


Figure 6. Metaphases from skin (*a*, *d*, *f*), brain (*b*), and spleen (*c*, *e*) cultures. (*a–d*) Diploid number; (*e*) near-tetraploid, 77; (*f*) hypo-16-ploid, about 280. Acetic orcein stain. (*a–e*) ×900, (*f*) ×460.

that viable cells with the number 39 did occur. Cells with 41 chromosomes were rare in the skin material, although some very clear instances were seen. The one pictured in FIGURE 5*b* had evidently undergone structural rearrangements, as evidenced by the single minute chromosome present.

The metaphase plate of FIGURE 5*j* is highly interesting. It was found in a culture of the sixteenth generation and had a unique structure: 88 chromosome arms, 74 of which formed telocentric chromosomes of the usual mouse type, and 14 of which were joined into metacentric chromosomes. The metacentrics were of different sizes, and the relationship between their arms varied, both submedian and subterminal locations of the centromeres occurring among them. Occasionally, metaphases with single metacentrics have been found in the present material, but no certain case with a regular occurrence of metacentric chromosomes has been encountered so far. It is well known, of course, that metacentrics may be present in the stemlines of some mouse tumors, so the change from telocentric to metacentric may occur as an evolutionary process. The fact that a single cell contains no less than seven metacentric chromosomes may indicate that the formation of this chromosome type is controlled by certain factors that acted somewhere in the ancestry of this cell. A random formation of metacentrics should have brought about their appearance in low frequency over the entire cell population, not a high frequency in only one individual cell.

Embryonic brain tissue. Several experiments were made with embryonic brain tissue, which grows well in the first generation, but more slowly in the second. We have made some attempts, so far unsuccessful, to obtain a third generation. Chromosome conditions are very clear in culture of brain tissue. Chromosome numbers were determined in 4 experiments, of which 3 (experiments 105, 110, and 111) were carried for 2 generations, although the chromosomes were followed through both generations only in experiment 105.

The chromosome numbers observed are presented in TABLE 10. One fact of special interest is seen from this table. In spite of the low number of chromosome number determinations there is no doubt that one of the experi-

TABLE 10
CHROMOSOME NUMBERS IN BRAIN CULTURES

Experiment number	Generation number	Chromosome number									Total cells examined	% ± 4x
		40	41	42	75	76	80	82	115	±160		
105	1	15	17	2	1	—	3	1	—	—	39	13
	2	9	14	—	—	1	6	—	1	1	32	25
110	2	11	—	—	—	—	2	—	—	—	13	18
111	2	10	—	—	—	—	2	—	—	—	12	20
208	1	11	1	—	—	1	5	—	—	1	19	32
Total........		56	32	2	1	2	18	1	1	2	115	20

ments, namely, experiment 105, follows a chromosome pattern of its own, distinct from those of the other experiments. As early as generation 1, experiment 105 exhibits a maximum at 2x + 1 chromosomes, and this maximum is maintained and even somewhat stressed in generation 2. Although this number was found in one cell of experiment 208, it is apparent that the 2x + 1 number is of significance only in experiment 105. Thus, it may be concluded that different cultures of the same tissue may take individual pathways for their genotypic variation, leading to diverging end results.

As compared with the embryonic skin cultures reported in the preceding section, the brain cultures behave characteristically in the following way. Tetraploidy plays a much more important role in the variation of skin cultures than in the brain tissue cultures. Although we have data from only 2 generations of the latter tissue, the difference from the corresponding generations of skin tissue is evident. In skin cultures the proportion of tetraploid mitoses started out in generation 1 with from 26 to 52 per cent of all mitoses, while in brain tissue cultures it averaged 20 per cent in the first generation. Although the diploid region held its own in our brain cultures, there is some indication of differences between the experiments; experiment 208 had a higher percentage of tetraploidy, and experiment 105 doubled its percentage of tetraploidy from generation 1 to generation 2.

It should be observed that, even though most established mouse tumors have stemlines in the hypotetraploid region, there are several that have a transitory period or settle permanently in the hyperdiploid region. It will be interesting to determine whether there is any correlation between the pattern of chromosome variation displayed in cultures of specific tissues and the final chromosome number region of transplantable tumors derived from these tissues. It may even be that the original proportion of diploid and polyploid cells in a tissue may influence its tendency toward development of malignancy. It would seem reasonable, for instance, that in the mouse the presence of a high fraction of tetraploid cells in a normal tissue would facilitate the development of malignancy, not only because of the closeness of tetraploidy to the optimal chromosome number region for cancer stemlines, but also because of the potentialities for chromosome variation inherent in the tetraploid number.

FIGURES *7a*, *7b*, *7c*, and *7e* picture the chromosomes of 3 cells with 41 chromosomes and 1 with 82 chromosomes, all from experiment 105. Evidence of structural variation is seen in *7a*, namely, the minute chromosome at 12 o'clock. The 3 plates with 41 chromosomes (*7a–7c*) represent progressive stages of colchicine action. FIGURE *6d* is a diploid metaphase of a brain cell.

Embryonic spleen tissue. Although several experiments were made with spleen tissue, chromosome studies were possible only in experiment 83, generations 1 and 2. The chromosome numbers counted are presented in TABLE 11, where the distribution of chromosome numbers is shown to be more of the brain type than of the skin type, as discussed above. The diploid region predominates, even though an increase of the tetraploid region is indicated from generation 1 to 2. Experimentation with 2x + 1 chromosomes is in process in the cultures of both generations. This is of some

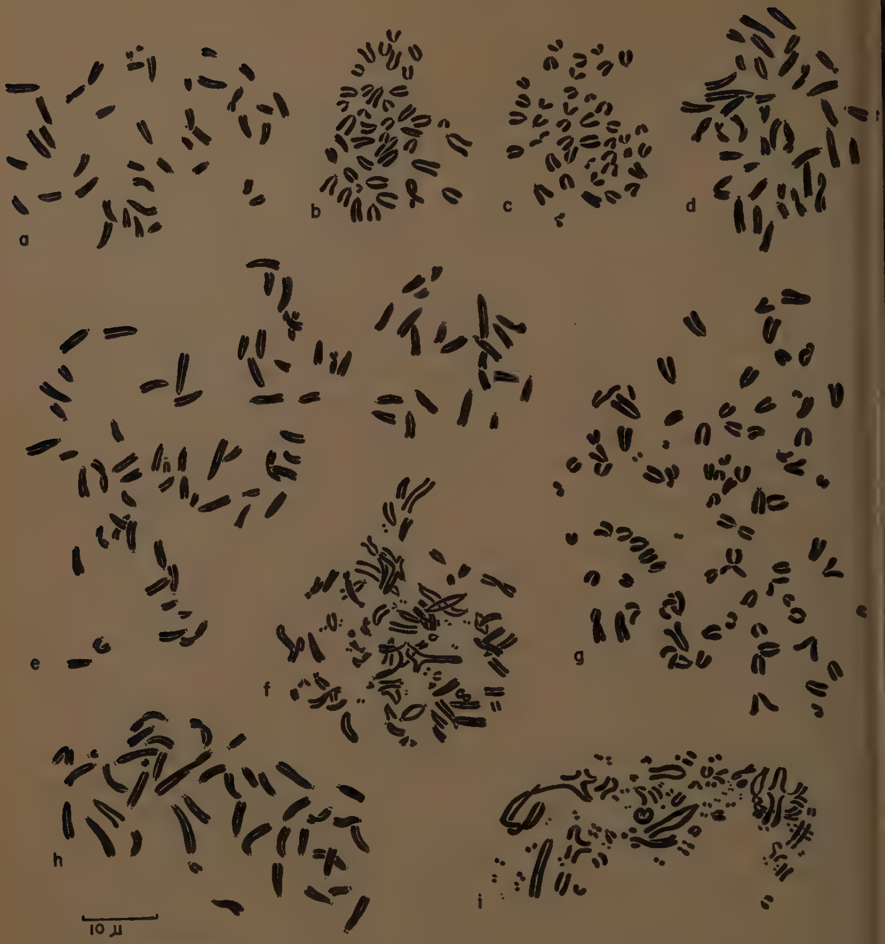


FIGURE 7. Metaphases of brain (*a–c, e*), spleen (*d, f, g, i*), and liver (*h*) cultures of generations 1 and 2. (*a–d, h*) Near-diploid numbers, 41, 41, 41, 40, and 42; (*e*) near-tetraploid number, 82; (*f, i*) near-tetraploid cells with extreme chromosome rearrangements (*i* is the same cell as FIGURE 2*n*). Acetic orcein stain. ×950.

interest in connection with the finding of Ford *et al.* (1957) that hyperdiploid stemlines are frequent in newly induced leukemias and reticulosarcomas.

FIGURE 7*d* shows a metaphase plate with 40 chromosomes, while FIGURES 7*f*, 7*g*, and 7*i* are approximately tetraploid cells—7*g* with a few indications of structural changes, 7*f* and 7*i* with quite excessive changes. FIGURE 7*g* contains 83 elements, one of which (at 10 o'clock) is a chromatid fragment, clearly cut off from a chromosome close by; another (at 9 o'clock, below the former) is a pair of acentric chromatid fragments. All the other elements are centric chromosomes, although some are smaller than any of the regular mouse chromosomes. FIGURES 7*f* and 7*i* are presented as instances of very

TABLE 11
CHROMOSOME NUMBERS IN ONE EXPERIMENT WITH SPLEEN TISSUE

Generation number	Chromosome number						Total cells examined	% ± 4x
	40	41	75	80	82	±160		
1	13	5	—	4	—	1	23	18
2	38	3	1	11	1	—	54	24
Total.	51	8	1	15	1	1	77	22

far-reaching structural rearrangements that may occur spontaneously in normal tissues grown *in vitro*. Both cells show evidence of extreme chromosome breakage at different stages of the preceding resting stage. Thus there are many, often quite small, acentric chromatid pairs. In FIGURE 7*f* there are strikingly many dicentric chromosomes. In addition to such presplit rearrangements, both cells have a great many very complex triradial and quadriradial configurations indicating chromatid exchanges, often involving several chromosomes. Some ring structures are also seen. The cell of FIGURE 7*i*, photographed in FIGURE 2*n*, is characterized by many very short chromosome pieces and, in addition, by extremely long elements that must represent combinations of several chromosome lengths. Our spleen cultures contained many cases of cells with similar extreme rearrangements. FIGURES 6*c* and 6*e* are photomicrographs of metaphases in spleen cells.

Embryonic liver tissue. Several experiments were set up with embryonic liver tissue. Usually they grew well and, in some cases, a second generation was successful. In spite of this we have managed to obtain little chromosome information. From 3 experiments, chromosome numbers were counted from a total of 21 cells. Of these counts, 29 per cent belonged to the tetraploid region. Within the diploid region some cells had 41 and 42 chromosomes. Of course, from these meager data, nothing can be said about the chromosome variation pattern of liver tissue. Because of the frequent occurrence in adult liver tissue of polyploid cells, it might be expected that tetraploid variation would be significant in liver cultures.

FIGURE 7*h* shows a 42-chromosome cell from a first generation. The 2 small chromosomes at 10 o'clock are new creations. One extremely long chromosome in the center of the plate indicates still more structural rearrangements in the ancestry of this cell.

The observations of this section show that the development of what will in due time become the karyotype of a cell strain stemline, or possibly of a cancer stemline, starts directly after the explantation of the normal cells *in vitro*. The mitotic irregularities frequently seen in the tissue culture evidently do not jeopardize the viability of some of the cells in which they occur. Therefore viable cells with changed karyotypes appear from the very beginning of a tissue culture.

The pattern of karyotypic changes is to a certain degree predetermined by the characteristics of the tissue. Each culture, however, represents an individual experiment in karyotype evolution. Considering the great number of factors involved in this process, it is probable that, should all the details of each karyotype be microscopically visible, two quite identical karyotypes from any established tissue culture cell strain or derivative cancer line would never be found.

Discussion

It is now well known that the development of cell strains continuously transferable *in vitro* may be associated with changes in the direction of malignancy. Moore (1957) reviews several recent cases in which cell strains of normal origin approach known malignant cell lines in some or all of the following qualities: cytological morphology, including chromosome constitution; growth rate; metabolic properties, especially the rate of anaerobic glycolysis; nutritional requirements; reactivity to viruses or chemicals; and capacity for malignant growth on inoculation *in vivo*.

As tissue cells adapt themselves to life *in vitro*, at the same time they may automatically acquire qualities typical of malignant cells. The environment *in vitro* favors the development of malignant traits. That the two adaptations are not identical, however, is evident from the fact that only a small fraction of tumors is capable of serial growth *in vitro* (Southam and Goettler, 1953; Southam, 1954; Fjelde, 1955).

The present investigation has given abundant and consistent evidence that genetic variation is at work in cultures of normal embryonic tissues. The variability starts immediately after the explantation and continues through later serial passages. Since it is made apparent only during mitosis, the genotypic experiments can be studied closely during the first and sometimes during the second generation, while mitoses are still frequent enough. During the ensuing period of sluggish growth, mitoses are scarce and the genotypic variation cannot be observed. However, there is every reason to assume that it is always in progress. Later on, after the formation of a cell strain, mitoses are again frequent, and it can be seen that, among other similarities to malignant tissue, the cell strain has evidence of chromosomal instability. The genetic variation, induced at the moment of explantation, of the embryonic tissue, continues steadily and leads directly into the variability well known in cancer tissue.

As stated in the introduction, our working hypothesis was that cancerogenesis is a selection process on the cellular level, favoring cells capable of autonomous growth. We shall now consider whether there is sufficient reason to assume a causal relationship between the genetic variability observed and the origin of malignancy.

For this issue it would have been most important to know unequivocally whether the mitotic mechanism shows any irregularities in the normal building up of the soma. Unfortunately, this must still be considered a point under discussion. During recent years several writers have claimed the presence of an excessive spontaneous aneuploidy in somatic tissues of various mammals. Their evidence, however, rests largely on squashed material, and it is well known that squashing easily causes the escape of one or more chromosomes from the metaphase groups. On the other hand, various workers who tried to avoid the risks of squashing have been unable to find any spontaneous aneuploid chromosome number variation in the materials studied by them. Thus, investigations by Sachs (1954), Walker and Boothroyd (1954), and Hungerford (1955) indicated a high degree of chromosome constancy in various somatic tissues of the mouse, rat, hamster, and man. J. H. Tjio and A. Levan (unpublished data) found no aneuploid variation in regenerating mouse and rat liver. In our opinion it is highly improbable that aneuploid or structural chromosome variation is of any significance in normal embryonic tissues. Therefore it seems safe to assume that the normal embryonic cells from which our cultures were set up were stable with respect to chromosome number and chromosome structure. By the change in environment involved in the explantation, this mitotic stability was upset, chromosome breaks were induced, and anaphasic irregularities appeared.

Mitotic irregularities have been observed in mammalian tissue cultures on many earlier occasions. Ludford (1953) reviews some cases, mostly induced by various treatments, but also some appearing spontaneously. Hsu (1952) and Hsu and Pomerat (1953) report the occurrence of aneuploid chromosome number variation in tissue cultures of various mammals. Hsu (1952) points out that this condition results from nondisjunction and not from structural changes: "no visible abnormalities such as lagging, bridge formation, uni- or multipolarization, were noticed."

This is in contradiction to the experience of one of us (J. J. B.), who has regularly found a low frequency of abnormal "postmetaphases" in the untreated control cultures of extensive chemotherapeutic screening experiments. Two such series of controls may be recalled here as instances of the frequency of such mitotic irregularities. Both series included embryonic mouse skin and Crocker mouse sarcoma 180. One of them (Biesele *et al.*, 1952) comprised skin cultures of 16 experiments with an average %AA of 4.9, and sarcoma 180 cultures of 24 experiments with 7.3% AA. The other series (Biesele, in press) included altogether 1930 and 2553 postmetaphasic mitoses for the skin and for the sarcoma 180, respectively, their %AA being 1.5 for the skin and 3.3 for the sarcoma. Thus, in both cases the normal tissue cells had a fair frequency of chromosomal variation not so much lower than that of the transplantable tumor.

The difference in absolute values of %AA between the two series is hardly surprising. They are both within the variation limits of the values found in the present experiments. Evidently environmental factors influence the frequency. In our experiments trypsin brought about a fivefold increase in %AA as compared with the mildest treatment possible. It seems that the zero value of %AA is possible only with cells *in situ*, and that as soon as they are explanted they start mutating. In this connection it should be mentioned that trypsin has been found earlier to have a deleterious action on cells in tissue culture. Thus, Neuman and McCoy (1955) report that the development of spindle shape in chick embryo cells *in vitro*, which is a sign of health in normal cultures, is counteracted by trypsin in concentrations as low as 5 μg./cc.

In our opinion the change in environment involved in the explantation of the tissue is the factor responsible for the mitotic irregularities. This settles the point raised in the introduction as to whether the mitotic disturbances begin before or after the onset of malignancy. Since the tissues in the present case are normal embryonic tissues, and since the first mitoses *in vitro* show irregularities, there can be no doubt that mitotic disturbances come before any induction of malignancy. The chromosomal irregularities cannot be merely symptoms of cells already cancerous, as is sometimes claimed.

It is a characteristic feature in the development of tissue cultures that an initial period of fairly good growth is followed by a longer or shorter period of sluggish growth. Evidently the cells bring along some substance or growth factor from the host body, this substance being gradually exhausted during the initial one or two generations. Consequently, the cells grow more and more slowly and nearly cease to propagate, often fading out completely during this latter period. It is conceivable that the random genotypic variation in process in all cultures will develop genotypes that respond differently to the *in vitro* conditions. These genotypes accumulate under constant genetic adjustment, and suddenly there may emerge a well-adapted type that is capable of vigorous growth *in vitro*. This genotype is recognizable microscopically by a new stemline karyotype, such as the hypotetraploid karyotype of the cell strain from experiment 76, or the hypertriploid karyotypes of various human cell strains (Levan, 1956a; Berman, *et al.*, 1957; Syverton, 1957). The genotypic variation has been the instrument by which this cellular adaptation is achieved. There is a causal relationship between the chromosomal variation as visible expression of the genotypic adaptation and the acquirement of the capacity for indeterminate life *in vitro*.

For this development it seems to be significant that a wide genotypic variation should be effected in the tissue culture as early as possible, during the initial period of moderate mitotic frequency in the first generations after explantation. After that period the creation of deviating genotypes occurs less often because of the scarcity of mitoses. That is probably why trypsinization acts favorably in the establishment of new cell lines. Trypsin treatment multiplies the initial frequency of aberrant cells. In any event, the period of sluggish growth, during which many new genotypes are tried, may lead to a period of vigorous growth, in which one of the genotypes developed eventually forms the stemline genotype for the new cell strain. We know that this change in growth habit is often connected with the acquisition of neoplastic capabilities. It is difficult to avoid the conclusion that these two qualities, capacity of independent growth *in vitro* and capacity of autonomous growth if inoculated *in vivo*, are both the result of the genotypic adaptation during the development of the tissue culture.

We have thus concluded that malignancy may originate in tissue culture by the selection of genotypic variants. Is there any evidence that a similar mechanism is acting when cancer develops *in vivo?* Certain facts support such a view. As would have been expected a priori, this is true especially of cancers induced by mutagenic agents, carcinogens, and irradiations. When a tumor is induced, for instance, by benzpyrene treatment, there is often a long period of latency between the induction and the development of full malignancy (Oberling, 1956). In our opinion this period corresponds to the early generations of tissue cultures, that is, to the period of genotypic experimentation. What is induced by the carcinogen is the initiation of genotypic instability, which much later may produce the complex cellular mutants capable of autonomous growth.

In the case of chemically induced cancerogenesis, mitotic irregularities have been observed during the latent period similar to those studied in tissue cultures in the present investigation (for example, Biesele and Cowdry, 1944; Ludford, 1953). It is interesting to recall, as pointed out by Ludford (1953), that most mitoses, after carcinogen treatment, are normal; only a low frequency of irregularities occurs. This suggests that the action of the carcinogenic agent is of a specific kind, different from the effect of such chemicals as c-mitotic substances, which act on all cells as long as the concentration is above a certain threshold value. The solitary irregularities appearing after treatment with carcinogens are signs of a permanent cellular shift toward less stable chromosome conditions. Only after a long period of trial and error do these irregularities eventually produce the genotypic changes necessary for starting autonomous growth. Similar patterns may be assumed for the induction of cancer by ionizing radiations.

Tumors started through hormonal disturbances are often hormone-dependent during early stages. If the stimulating hormonal situation is relieved, the tumor may regress. However, if the dependent growth has continued for a length of time, it may become hormone-independent (cf. Furth, 1953). Perhaps this change corresponds to the appearance of a new genotype that grows autonomously.

Generally speaking, precancerous stages preceding many types of malignant neoplasms are stages of mitotic activity combined with mutagenic chromosome changes. Such conditions, if not interrupted, sooner or later may lead to malignancy, namely, when a suitable constellation of mutations has been accumulated in one cell. As long as autonomy has not been attained in any cell, the tumor may regress if the factors governing the precancerous condition are abolished, because surrounding normal cells still have selective advantage when normal environmental conditions are restored. Once the genotype capable of autonomous growth has been formed, the change is irreversible.

Naturally we are not of the opinion that this mechanism, involving a thorough reorganization of the entire genotype, would be the only way in which malignancy may become established. On the contrary, many facts indicate that neoplasia may develop along different pathways. The mechanism illustrated by the present investigation, that is, the origin of malignant properties in normal cells grown *in vitro*, works with an excessive random mutative experimentation. This mechanism, as suggested above, may be of significance especially in cancers induced by mutagenic agents. This mechanism also acts during cancer progression and may well be thought to act accordingly in all kinds of cancers, irrespective of their mode of origin.

On the other hand, the viral or hormonal induction of cancer may follow different chromosomal patterns. Certain facts indicate that gross chromosomal reorganization may be unnecessary at their induction. Preliminary experiments with a virus-induced mouse leukemia* (cf. Friend, 1957), showed that the chromosome number 40 predominates in all cancer cells; no signs of any structural rearrangements were observed. If genotypic changes are involved in the induction of a neoplasm of this type, they must be different from those observed in the present investigation. The regularity and the rapidity with which certain virus tumors are induced would be understandable if the virus were capable of influencing certain loci of the genotype, namely, those necessary for normal coordinative growth, in a kind of directed mutation process, perhaps of the transduction type, as opposed to the random mutation characteristic of the development of malignancy *in vitro*.

It is interesting to note that a sample of normal embryonic tissue, which ordinarily, *in situ*, would have shown no tendencies toward cancer development may, if explanted and maintained *in vitro*, immediately start a development that, within a few months, leads to the establishment of a permanently growing cell line, often with malignant properties. This condition suggests that the host body, as such, normally has an influence on its tissues that opposes cancer origin. This rather vague statement may be interpreted in the following terms of population genetics. Any somatic cell is genetically adapted to its environment within the tissue, and only here is it capable of optimal performance of its life processes, including cell propagation. If a cell with deviating genotype appears in an embryonic tissue, the odds are that it will become outgrown by the normal cells surrounding it. Only if conditions are essentially changed in the body may cells with deviating genotype have a selective advantage *in situ*, and only then will a genotypic variability lead to cancer growth.

Explanted *in vitro*, the same embryonic tissue finds itself under highly different conditions. The normal genotype is barely capable of survival and of going through one or a few mitoses. Even for this it depends on the aid of substances brought along from the host body. Under these circumstances there is a good chance that deviating genotypes, which are steadily produced in abundance because of the explantation "shock," will include some that are just a little superior to the normal genotype, a little more capable of carrying out mitosis in these abnormal surroundings. A strong selection pressure will act toward the accumulation of such mutants and gradually adjust them to ever better performance in the *in vitro* conditions.

In conclusion, our observations give strong support to the somatic mutation theory of cancer. Previously, most critical evidence for this theory has been derived from greatly advanced cancers, such as transplantable ascites tumors. Therefore, the possible role of chromosome variability at the induction of malignancy has been inferred only theoretically, and conclusive evidence has been missing on the decisive point of which comes first, the malignancy or the chromosomal disturbance. Are the two perhaps parallel but independent phenomena?

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* Data provided by Charlotte Friend of the Sloan-Kettering Institute for Cancer Research, New York, N. Y.

In our experiments we demonstrated that normal embryonic cells started mutating immediately on their explantation *in vitro*, while no signs of malignancy appeared until much later. Therefore, in our case there can be no doubt that the chromosomal irregularities precede the malignancy; and there s strong evidence for a causal relation between the two phenomena.

In our opinion, the capacity for at least certain types of cancerous growth nvolves a genotypic adaptation toward cellular autonomy. This adaptation s the result of selective processes within a heterogeneous cell population. The heterogeneity underlying this selection may be caused in different ways: by a change in the cellular environment, as for instance by explantation *in vitro;* or by physicochemical mutagenic agents, either extrinsic, such as ionizing radiation or carcinogenic chemicals, or intrinsic, such as hormonal or metabolic changes. The main factors favoring the induction of malignancy in a tissue are high incidence of chromosome breakage together with high mitotic activity.

Summary

Cells of normal tissue origin maintained in serial culture may give rise to cell lines capable of continuous growth *in vitro*. The lines may exhibit new properties and may approach malignant cell lines in their qualities. Established cell lines, both of normal and of cancerous origins, have similar chromosomal behavior: (1) the mitotic mechanism shows instability, with variations in chromosome number and structure, and (2) the stemline chromosome set differs from the normal karyotype of the species.

Following successive passages of cultures of a few mouse tissues, we found that the chromosomes of normal embryonic cells start varying immediately after their explantation. One factor determining the extent of chromosome variation is the original treatment of the tissue. Thus, cells separated by trypsinization show a higher incidence of chromosome breaks than those mechanically minced.

Our cultures of embryonic skin began mainly with the normal diploid chromosome set and a fraction of tetraploid cells. Diploid mitoses decreased in number at each transfer until, in one case, hypotetraploid cells took over completely at about the tenth transfer, and the culture started growing vigorously in the manner of established cell lines. By transfer 22, implantation of the cultured cells into mice produced spindle cell sarcomas. Other tissues followed different patterns. Some brain and spleen cultures showed extensive hyperdiploidy, but have not yet formed easily transferable cell lines.

The drastic environmental change involved in explantation *in vitro* of an embryonic tissue is evidently responsible for inducing chromosomal instability. The deviating genotypes compete as new stemlines until one that is well adapted to continuous life *in vitro* comes into supremacy.

The chromosomal changes in this adaptation may be regarded as model experiments for chromosome behavior during the development of malignancy

in vivo, which could be interpreted as depending on opportunity for geno typic variation in a proliferating tissue in an environment under which the normal genotype suffers selective disadvantage.

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CHEMICALLY INDUCED IMITATIONS OF MITOTIC ANOMALIES COMMON TO CANCER CELLS*

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Introduction

Among the conditions suggested by A. Levan, elsewhere in these pages, as conducive to the development of malignancy in mammalian cells was a mutating cellular population, detected cytologically by chromosomal breakage, rearrangement, and gain or loss. It was also pointed out that the cells of frank neoplasms constitute such mutating populations, and that in tissue cultures of Crocker mouse sarcoma 180, for example, there is a spontaneous incidence of abnormal postmetaphasic mitotic figures ("AA") with evidence of chromosomal disturbances of the order of approximately 10 per cent. In some experiments this spontaneous incidence is higher. Fresh explants of embryonic mouse skin commonly show a lower frequency of such abnormalities. Both in the sarcoma 180 cultures and in the embryonic skin cultures the frequencies of these abnormalities can be changed by treatment with certain chemicals. Differences in the responses of the sarcoma cells and the embryonic fibroblasts to various antimetabolites, or their combinations with normal metabolites, suggest some differences in the mechanisms at work in the production of chromosomal disturbances.

Materials and Methods

The cells used in these studies were of primary explants of Crocker mouse sarcoma 180 and embryonic mouse skin (especially the fibroblasts) cultivated in a chicken plasma clot under a "classic" medium of serum, embryo extract, and balanced salt solution. The cultures were planted on coverslips in flattened roller tubes. After 2 days' incubation at 37° C., the cultures were exposed for 24 hours to selected chemicals added to their medium. They were then fixed in alcohol-acetic acid, stained with the Feulgen nuclear reaction, counterstained with light green, and subjected to microscopic examination. The mitoses studied were in the outgrowths of the cultures; those in the original explants were disregarded.

The chemicals used had been submitted to the Division of Experimental Chemotherapy of the Sloan-Kettering Institute or were commercial specimens.

Results and Discussion

It was hoped that this study would contribute to the answers to two questions: (1) what is the mechanism by which chromosomes undergo

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bridging and fragmentation, and why is there a significant amount of this n tissue cultures, ordinarily greater in cultures of mouse sarcoma 180 than n cultures of embryonic mouse skin? (2) From what does the background rate of spontaneous chromosomal disturbances in tissue culture arise, particularly in the absence of trypsinization?

It has often been proposed that disturbances in nucleic acid metabolism underlie some chromosomal aberrations. If so, it may readily be expected that interference in nucleic acid purine biosynthesis by such antiglutamine agents as O-diazoacetyl-L-serine—azaserine (Skipper *et al.*, 1954; Hartman *et al.*, 1956)—and 6-diazo-5-oxo-L-norleucine—DON (Buchanan, 1957)—should damage chromosomes. It will be recalled that azaserine is a potent bacterial mutagen (Hemmerly and Demerec, 1955). In mouse tissue cultures, both azaserine and DON cause chromosomal damage.

TABLE 1

EFFECTS OF O-DIAZOACETYL-L-SERINE (AZS) AND 6-DIAZO-5-OXO-L-NORLEUCINE (DON) ON THE MITOSES OF MOUSE SARCOMA 180 AND EMBRYONIC MOUSE SKIN FIBROBLASTS IN TISSUE CULTURE

Treatment	Metaphases/prophases		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	Sarcoma 180	Fibroblasts	Count	Per cent aberrant	Count	Per cent aberrant
Control	0.6	0.6	335	5	215	1
AZS 0.01 mM	0.3	0.6	449	14	252	2
AZS 0.1 mM	0.3	0.4	28	18	352	4
Control	0.9	0.4	564	6	444	1
DON 0.0001 mM	0.7	0.6	285	10	145	1
DON 0.001 mM	0.4	0.7	322	19	245	2
DON 0.01 mM	0.3	0.7	268	19	82	7
DON 0.1 mM	0.5	0.9	70	40	114	5

TABLE 1 gives data for this effect. Exposure for 24 hours to 0.1 mM azaserine (that is, 0.1 μM/ml.) increased by several times the proportion of aberrant postmetaphasic figures with chromosomal bridges, chromosomal fragments, or micronuclei. Less effect was seen at 0.01 mM azaserine, and none was obvious at 0.001 mM. The drop in ratio of metaphases to prophases seen on treatment with azaserine may result from a comparative prolongation of prophase. Perhaps this is related to interference in ribonucleic acid, an important synthesis of which takes place just before prophase (Taylor and McMaster, 1954). The susceptibility of intracellular ribonucleic acid to ribonuclease treatment probably accounts for the prophase poisoning by this enzyme (Firket *et al.*, 1955).

DON was considerably more active than azaserine, both in the extent o chromosomal damage, which affected up to 40 per cent of postmetaphasi figures in the sarcoma cells, and in the low concentrations to which effectiveness extended. The greater activity of DON may be attributed in part t its inhibition of a step in purine biosynthesis before the step resulting in th formation of formyl glycinamidine ribotide, this latter step being notably blocked by azaserine and also by DON (Moore and LePage, 1957).

However, azaserine and DON also differed in other respects. The metaphase/prophase ratio, for instance, fell in sarcoma 180 cells treated with DON, but rose in treated fibroblasts. Moreover, the relative mitotic inhibition, greater in sarcoma cells than in fibroblasts under treatment with azaserine (Biesele, in press), was inverted when the cultures were instead treated with DON (FIGURE 1). Perhaps these differences from azaserine are related to the interference DON produces in nucleic acid pyrimidine metabolism (M. Eidinoff, personal communication).

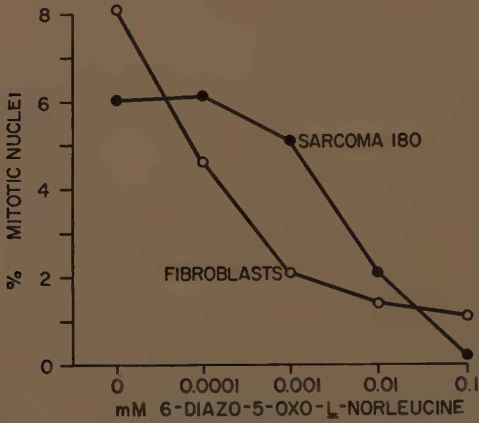

FIGURE 1. Mitotic incidence of sarcoma 180 cells and embryonic mouse skin fibroblasts after 24 hours' exposure to various concentrations of DON *in vitro*.

That the action of azaserine in damaging chromosomes is related to its status as an antagonist of glutamine is indicated in TABLE 2. Simultaneous administration of L-glutamine and azaserine to mouse tissue cultures prevented the increase in aberrant postmetaphases seen with azaserine alone. It is important to note that 2 mM L-glutamine alone added to the medium did not eliminate the background of spontaneous postmetaphasic aberrations either in the sarcoma cells or in the embryonic fibroblasts.

The greater effectiveness of both azaserine and DON in producing chromosomal aberrations in cells of sarcoma 180 than in fibroblasts of embryonic mouse skin *in vitro* probably represents the comparative importance o *de novo* synthesis of nucleic acid purines in the two cell types. Bennett *et al.* (1955) have remarked on the greater tendency of animal tumors t synthesize their nucleic acid purines than to incorporate preformed purines Sarcoma 180 was one of a number of animal tumors that incorporated relavely less of labeled guanine and adenine, but more of 2,6-diaminopurine, to their combined nucleic acids than did the host intestine or liver, and latively much more of labeled formate than did the host liver. Sarcoma 80 was one of the least active of the four strains of mouse ascites tumors sted by LePage and Sartorelli (1957) for their *in vivo* rates of incorporation labeled adenine into nucleic acid adenine. Besides the possible differences etween sarcoma 180 cells and embryonic skin cells in the relative importance *de novo* synthesis and the incorporation of preformed purines, it is also dvisable to consider the possibilities of differences between cell types in the xtents or the rates of uptake of azaserine or DON (Jacquez, 1957).

If embryonic mouse skin fibroblasts are more ready to incorporate exogeous purines into chromosomal nucleic acid than are sarcoma 180 cells, one

TABLE 2

NTAGONISTIC EFFECT OF L-GLUTAMINE ON POSTMETAPHASIC ABERRATIONS PRODUCED BY AZASERINE IN MOUSE TISSUE CULTURES

Treatment	Postmetaphases			
	Sarcoma 180		Fibroblasts	
	Count	Per cent aberrant	Count	Per cent aberrant
ontrol	100	13	100	6
zaserine 0.1 mM	21	43	196	10
-Glutamine 2.0 mM	147	8	100	8
zaserine 0.1 mM + L-glutamine 2.0 mM	100	9	135	4

night expect that blocking *de novo* synthesis of nucleic acid purines and at he same time making available an incorporable purine analogue might result n greater chromosome breakage in the fibroblasts.

TABLE 3 gives data from an experiment in which this seems to have ccurred. Especially with the combination of 0.01 or 0.001 mM azaserine nd 1.0 mM 6-mercaptopurine, chromosome breakage in the fibroblasts ncreased greatly. With this combination treatment, breakage in the sarcoma cells did not exceed the amount produced by the 6-mercaptopurine lone. The results suggest that there are optimal combinations of azaserine nd 6-mercaptopurine for skin chromosome breakage, and that 0.1 mM zaserine was too great for maximal effect. Unfortunately, 0.1 mM azaserine lus 6-mercaptopurine produced such a pronounced preprophasic inhibition f mitosis in the sarcoma cells that no assessment of chromosome breakage was possible. This table also gives evidence of a conflict between the effects f azaserine and 6-mercaptopurine on the metaphase/prophase ratio. At .1 mM, azaserine decreased the ratio, but at lower concentrations of azaerine, 1.0 mM 6-mercaptopurine succeeded in raising the ratio.

TABLE 3
MITOTIC EFFECTS OF AZASERINE (AZS) AND 6-MERCAPTOPURINE (6MP) IN COMBINATION ON MOUSE TISSUE CULTURES

Treatment	Metaphases/ prophases		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	Sarcoma 180	Fibro-blasts	Count	Per cent aberrant	Count	Per cent aberrant
Control	1.3	0.6	737	10	451	1
6MP 1.0 mM		1.3	265	20	473	22
6MP 0.1 mM	0.7	0.9	144	6	105	8
AZS 0.1 mM + 6MP 1.0 mM		0.3			267	15
AZS 0.1 mM + 6MP 0.1 mM		0.4			167	22
AZS 0.01 mM + 6 MP 1.0 mM	1.2	1.3	194	17	295	58
AZS 0.01 mM + 6MP 0.1 mM	0.6	0.5	218	10	233	10
AZS 0.001 mM + 6MP 1.0 mM			72	13	123	49
AZS 0.001 mM + 6MP 0.1 mM		1.1			75	15

Another glutamine antagonist is its derivative, gamma-glutamyl hydrazide. It produced a selective mitotic inhibition of sarcoma 180 cells (FIGURE 2). Data from an experiment are given in TABLE 4. As with azaserine, the proportion of aberrant postmetaphases with chromosomal disturbance increased on treatment with gamma-glutamyl hydrazide. L-Glutamine successfully antagonized this effect, but adenine sulfate did not. The suggestion of an increase in damage with added adenine in the skin fibroblasts may be real, for adenine, especially in excess, is known to be a chromosome-breaking agent (Kihlman, 1950; Biesele *et al.*, 1952). In this case one might speculate that the presence of the glutamine antagonist made for a greater incorporation of adenine in the fibroblasts, as with 6-mercaptopurine in the presence

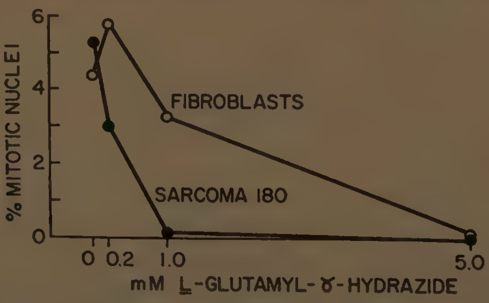

FIGURE 2. Mitotic incidence of sarcoma 180 cells and embryonic mouse skin fibroblasts after 24 hours' exposure to various concentrations of gamma-glutamyl hydrazide *in vitro*.

f azaserine. The combination made mitoses rare in the sarcoma 180 ultures.

Analogues of physiological purines have been of interest slightly longer han have antiglutamines, but only recently has it become possible, with he synthesis of purine riboside-5′-phosphate (Magrath and Brown, 1957), to xamine the mitotic effects of the series—purine, nucleoside, and nucleotide—ith other than the physiological substances. TABLE 5 presents the results f tests with this series, starting with the free base, unsubstituted purine. s previously reported (Biesele *et al.*, 1955), unsubstituted purine is differntially toxic to sarcoma 180 cells in culture, and differential mitotic inhiition from 24-hour exposure extends down through 0.05 mM. Chromoomal damage from purine is evident in embryonic skin fibroblasts at 0.1 mM and in sarcoma 180 cells at 0.01 mM. There is less differential toxicity from 9-β-D-ribofuranosylpurine or its 5′-phosphate, which agree closely in this respect, but it is necessary to drop their concentrations to 0.005 mM to find appreciable numbers of mitoses. About half the postmetaphasic mitotic figures in both sarcoma cells and fibroblasts were aberrant at 0.005 mM of the nucleoside or the nucleotide. At 0.001 mM, abnormalities were still present in the sarcoma postmetaphases, but the fibroblasts were at control levels.

TABLE 4

FFECTS OF L-GLUTAMINE AND ADENINE SULFATE ON POSTMETAPHASIC ABERRATIONS PRODUCED BY GAMMA-GLUTAMYL HYDRAZIDE (GGH) IN MOUSE TISSUE CULTURES

Treatment	Postmetaphases			
	Sarcoma 180		Fibroblasts	
	Count	Per cent aberrant	Count	Per cent aberrant
Control	311	28	100	3
GGH 0.5 mM	69	68	100	20
GGH 0.5 mM + L-glutamine 2.0 mM	100	13	100	5
GGH 0.5 mM + adenine 1.0 mM	17	70	100	40

The ratio of metaphases to prophases increased in all cases about parallel with the extent of postmetaphasic aberrations, with the exception of the fibroblasts treated with 0.005 mM purine riboside. Here a prophasic delay was evident. The increases in the ratio reflected delays in passing through metaphase. It may be recalled that slowness in passing through metaphase, considered by Koller (1947) to be characteristic of cancer cells (a contention disputed by Fardon and Prince, 1952), was attributed by Lettré (1952) to a relative deficiency in adenosine triphosphate, which was considered necessary for spindle function. Perhaps in our cases it results from the formation of purine riboside polyphosphates, which Gordon and Brown (1956) foun to be produced from purine riboside in the rat, and their antagonism of th action of adenosine triphosphate. In this respect it is highly suggestiv that the effectiveness in increasing the metaphase/prophase ratio, especiall as seen in the sarcoma cultures, was greatest for the phosphate, intermediat for the riboside, and least for the free purine.

It is perhaps true that other cytological effects of unsubstituted purin and other analogues of physiological purines are mediated at least in part b the formation of their nucleoside polyphosphates. These would be the fina precursors to be expected in their incorporation into nucleic acids, if the

TABLE 5
EFFECTS OF PURINE, PURINE RIBOSIDE (PR), AND PURINE RIBOSIDE-5′-PHOSPHATE (PR
ON THE MITOSES OF MOUSE SARCOMA 180 AND EMBRYONIC MOUSE
SKIN FIBROBLASTS IN TISSUE CULTURE

Treatment	Metaphases/prophases		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	Sarcoma 180	Fibroblasts	Count	Per cent aberrant	Count	Per cent aberrant
Control	0.6	0.3	237	19	293	4
Purine 0.1 mM	(none)	0.6	(no mitoses)		247	19
Purine 0.05 mM	(few)	0.6	(few mitoses)		339	4
Purine 0.01 mM	1.0	0.5	154	53	228	2
PR 0.005 mM	2.2	0.3	161	55	71	56
PR 0.001 mM	1.6	0.7	242	41	412	4
Control	0.8	0.5	214	36	239	2
PRP 0.005 mM	2.8	1.0	200	53	83	42
PRP 0.001 mM	2.1	0.5	172	44	138	6

follow patterns of synthesis determined by Grunberg-Manago *et al.* (1955 for ribonucleic acids and by Kornberg *et al.* (1956) for desoxyribonucleic acids If incorporation of a purine analogue into nucleic acid interferes in hydroge bonding (Lasnitzki *et al.*, 1954) with the base in the complementary helica chain (Watson and Crick, 1953), perhaps chromosome breaks will result It is of great interest to note that Kihlman (1955a) found that chromosom breakage in onion root tips by 8-ethoxycaffeine required the presence o aerobic conditions such that respiration could furnish compounds containin energy-rich phosphate bonds. The same held true for chromosomal sticki ness with this agent, and the necessary compound was postulated to be AT (Kihlman, 1955b). A similar explanation may apply to Novick's (1956 finding that anaerobic conditions reduced the mutagenicity of free purines t bacteria growing in a chemostat.

Finally, the expected antagonism between purine analogue nucleoside olyphosphates and adenosine triphosphate in the support of certain neces-ary syntheses preliminary to cell division would account for some part of he prevention of entry into mitosis. It is generally agreed that the support-ng metabolism for cell division needs some sort of energy carrier and, in any cases, this appears to be adenosine triphosphate.

The actions of some purine analogues may be more complex, however; erhaps they interfere in the actions of other physiologically important ucleotides. Thus, 6-mercaptopurine and coenzyme A exhibit some mutu-lly antagonistic effects on incidence of mitosis (Biesele, 1954) and on lipo-enesis and mitochondrial morphology (Biesele, 1955). 6-Mercaptopurine was also demonstrated to interfere in the synthesis of diphosphopyridine nucleotide in mice (Kaplan *et al.*, 1956). Fernandes *et al.* (1956) suggested that 6-mercaptopurine might affect purine coenzymes, because it depressed incorporation of glycine into acid-soluble purines of certain mouse tumors. The fact that 6-mercaptopurine depressed the incorporation of glycine into nucleic acid purines of four mouse ascites tumors (Greenlees and LePage, 1956) must not be overlooked, for this means that it may influence the same over-all pathway of purine biosynthesis affected by antiglutamines. The data of our TABLE 3 should be examined in this light.

TABLE 6
ANTAGONISM OF MITOTIC EFFECTS OF THIOGUANINE (TG) BY COENZYME A (CoA) IN MOUSE TISSUE CULTURES

Treatment	Metaphases/prophases		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	Sarcoma 180	Fibroblasts	Count	Per cent aberrant	Count	Per cent aberrant
Control	0.9	0.4	194	3	120	2
TG 0.1 mM	1.4	1.0	37	95	26	69
TG 0.1 mM + CoA 0.2 mM	0.5	0.2	73	48	104	30

The antagonism between 6-mercaptopurine and coenzyme A extends to chromosome breakage (Biesele, in press). This is also true, in more limited measure, for the related agent 2-amino-6-mercaptopurine, or thioguanine (TABLE 6).

TABLE 7 presents data from an experiment with 6-mercaptopurine and the two closely related factors, coenzyme A and α-lipoic acid. Some of the fibroblast data given here have been included in a partial report of the experiment (Biesele, in press). It is seen that, at 0.2 mM, coenzyme A blocked the development of postmetaphasic aberrations in both sarcoma cells and skin fibroblasts treated *in vitro* with 1.0 mM 6-mercaptopurine. The effect of α-lipoic acid, however, was to increase aberrations from 6-mercaptopurine in fibroblasts, while it brought them to control levels in sarcoma 180 cells. Alone, coenzyme A at 0.2 mM or α-lipoic acid at 0.2 mM did not strikingly decrease the background rate of aberrations in sarcoma 180 cells, but there may have been a significant reduction with the two together.

TABLE 7

EFFECTS OF COENZYME A AND DL-α-LIPOIC ACID ON POSTMETAPHASIC ABERRATIONS PRODUCED BY 6-MERCAPTOPURINE (6MP) IN MOUSE TISSUE CULTURES

Treatment	Postmetaphases			
	Sarcoma 180		Fibroblasts	
	Count	Per cent aberrant	Count	Per cent aberrant
Control	214	19	277	1
6MP 1.0 mM	83	25	219	11
6MP 1.0 mM + coenzyme A 0.2 mM	227	6	232	1
6MP 1.0 mM + lipoic 0.2 mM	168	19	138	31
6MP 1.0 mM + lipoic 0.2 mM + coenzyme A 0.2	303	4	164	2
Lipoic 0.2 mM + coenzyme A 0.2 mM	241	9	177	2
Coenzyme A 0.2 mM	278	13	259	1
Lipoic 0.2 mM	147	18	288	2

Not only α-lipoic acid, but also insulin had this peculiar effect of enhancing chromosomal damage (and also preprophasic mitotic inhibition) from 6-mercaptopurine in mouse fibroblasts (Biesele, in press). It should be recalled that lipoic, or 6,8-dithiooctanoic acid, and insulin have certain structural features in common.

This same inverse effect on fibroblasts and sarcoma 180 cells was produced by insulin and thioguanine (TABLE 8). This table also includes data from an experiment with thioguanosine, or 2-amino-6-mercapto-9-β-D-ribofuranosylpurine (Fox *et al.*). The data indicate that thioguanine increased chromosomal damage in both sarcoma 180 cells and embryonic skin fibroblasts. Insulin modified this toward less damage in the sarcoma cells and much more in the fibroblasts. On both tissues, thioguanosine alone was much more active than thioguanine alone, but insulin had little effect on its activity. Changes in the metaphase/prophase ratio paralleled the changes in proportion of aberrant postmetaphases.

The companion nucleoside of 6-mercaptopurine, or 6-mercapto-9-β-D-ribofuranosylpurine, is also available (Johnson and Thomas, 1956) and has been studied for chromosome breakage. TABLE 9 gives the frequency of

TABLE 8

INFLUENCE OF INSULIN ON MITOTIC EFFECTS OF THIOGUANINE (TG) AND OF THIOGUANOSINE (TGR) ON SARCOMA 180 CELLS AND FIBROBLASTS OF EMBRYONIC MOUSE SKIN IN TISSUE CULTURE

Treatment	Metaphases/prophases		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	Sarcoma 180	Fibroblasts	Count	Per cent aberrant	Count	Per cent aberrant
Control	0.6	0.6	154	16	96	2
TG 0.05 mM	1.2	0.7	80	28	246	9
TG 0.05 mM + insulin 1 unit/ml	0.5	1.0	88	11	181	48
Control	0.6	0.3	237	14	293	4
TGR 0.05 mM	1.8	0.8	75	79	194	35
TGR 0.05 mM + insulin 1 unit/ml	1.6	1.0	74	70	158	32

TABLE 9

EFFECTS OF 6-MERCAPTOPURINE RIBOSIDE (6MPR) ON THE MITOSES OF MOUSE SARCOMA 180 AND EMBRYONIC MOUSE SKIN FIBROBLASTS IN TISSUE CULTURE

Treatment	Postmetaphases			
	Sarcoma 180		Fibroblasts	
	Count	Per cent aberrant	Count	Per cent aberrant
Control	400	13	400	2
6MPR 0.001 mM	202	12	350	1
6MPR 0.01 mM	121	19	188	4
6MPR 0.1 mM	357	26	291	9
6MPR 1.0 mM	78	55	370	28
6MPR 4.0 mM	(few mitoses)		122	49

aberrant postmetaphasic mitoses at various concentrations of 6-mercaptopurine riboside.

This riboside was considerably more active than its free purine in causing chromosome breakage. The activity was more vigorously expressed against sarcoma 180 cells than against fibroblasts at any concentration.

Diverse physiological agents were tested (TABLE 10) against 6-mercaptopurine riboside. Coenzyme A produced only a slight release of the mitotic inhibition of sarcoma 180 cells and successfully antagonized postmetaphasic aberrations in fibroblasts, but not in sarcoma cells. Insulin was without effect on the action of 6-mercaptopurine riboside, just as it failed to influence thioguanosine (TABLE 8). Adenosine triphosphate did not help the sarcoma cells, and it caused a decreased mitotic incidence in fibroblasts while, at the same time, it eliminated most of their chromosomal breakage. This suggests that preprophasic inhibition and chromosomal damage by 6-mercaptopurine riboside are not brought about by the same or by very closely linked mechanisms. Diphosphopyridine nucleotide ameliorated both mitotic inhibition and chromosomal damage for sarcoma 180 cells; it also greatly improved the postmetaphases for the fibroblasts.

TABLE 10

INFLUENCE OF VARIOUS PHYSIOLOGICAL AGENTS ON MITOTIC EFFECTS PRODUCED BY 1.0 MM 6-MERCAPTOPURINE RIBOSIDE (6MPR) ON SARCOMA 180 CELLS AND FIBROBLASTS OF EMBRYONIC MOUSE SKIN IN CULTURE

Treatment	Mitotic Cells		Postmetaphases			
			Sarcoma 180		Fibroblasts	
	S180 (%)	Skin (%)	Count	Per cent aber.	Count	Per cent aber.
Control	5.0	5.5	300	16	458	1
6MPR	0.3	4.3	44	48	221	32
6MPR + coenzyme A 0.2 mM	1.4	4.1	146	51	264	3
6MPR + insulin 1 unit/ml	0.1	3.8	31	48	249	36
6MPR + adenosine triphosphate 1.0 mM	0.4	1.5	37	68	116	4
6MPR + diphosphopyridine nucleotide 1.0 mM	4.3	3.0	124	34	178	2

Preliminary data have been obtained with a number of other physiological compounds containing purines. They indicate that hypoxanthine and, to a lesser extent, inosine, tend to antagonize 6-mercaptopurine riboside as does diphosphopyridine nucleotide. Adenine, adenosine, and several adenylic acids have effects approximating a position intermediate between those given in the table for coenzyme A and for adenosine triphosphate. In this experimental set, neither coenzyme A alone nor adenosine triphosphate alone decreased postmetaphasic aberrations from the control level in sarcoma 180 cells, although each made for higher mitotic incidence. Adenosine triphosphate alone even increased chromosomal bridging and fragmentation in sarcoma 180 cells.

Summary and Conclusions

In an attempt at synthesis of these data, one is struck by the differences in response of the sarcoma 180 cells from the response of embryonic mouse-skin fibroblasts with respect to chromosome breakage. There is the enhancement of breakage from the mercaptopurines in the fibroblasts, but not in the sarcoma cells, by treatment with the disulfide agents, insulin, or α-lipoic acid. There is also the prevention of breakage from the nucleoside in fibroblasts by coenzyme A, adenosine triphosphate, or various adenine compounds, but the failure of this to occur in sarcoma cells. This situation exists despite amelioration by coenzyme A of breakage from the free purines in both sarcoma cells and fibroblasts.

One key to the solution of this multifactorial complex may lie in the relative competence of mitochondria in the two cell types. Do the mitochondria of both have a 6-mercaptopurine-sensitive glycolytic system, as Hochstein (1957) described for some tumor cell mitochondria? Are the sarcoma 180 cells insensitive to insulin? Following Burk and Woods (1956), we should expect that sarcoma 180 cells would exhibit a high glycolysis, little restrained by hexokinase-insulin inhibitors. Another key may lie in the relative uptakes of antimetabolites by the two cell types. Another probably lies in the possible difference in relative importance of the biosynthetic pathway to nucleic acid purines and the pathway involving incorporation of preformed exogenous purines. It is difficult to avoid inferring from the data that the sarcoma 180 cells depend largely on *de novo* purine synthesis and that their ability to incorporate exogenous purines is much more limited than that of the embryonic skin fibroblasts. Perhaps the reaction with nucleosides is different.

Do these results and inferences with antimetabolites have any implications for an understanding of the spontaneous chromosome breakage in tissue cultures, especially of neoplastic cells? They may have, for in effect the presence of an antimetabolite is equivalent to a deficiency of the normal metabolite and may produce derivative deficiencies of other materials that represent later intermediates in inhibited pathways. Conversely, this also means, in cases where a balance of related but different monomers (if we may call them such) is needed for proper synthesis of linearly differentiated polymers (we refer to amino acids and proteins, to nucleotides and nucleic acids), that a possible excess of complementary materials may exist. It may be significant, for instance, with reference to chromosome breakage by adenine, that guanine was found to be incorporated poorly into nucleic acids in a series of animal tumors, relative to control tissues of the host animals (Bennett *et al.*, 1955). Perhaps the background chromosome breakage could be reduced to a minimum in tissue cultures by exactly the right balance of nutrients, but if a situation such as this poor incorporation of guanine characterizes cancer cells, it might be impossible to eliminate, on a nutritional basis, all chromosome breakage from cancer cell cultures. If the poor guanine incorporation had a genetic basis, the cells, under the provided conditions, would have a genetically determined chromosomal and genetic instability.

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CELLULAR ASPECTS OF TUMORIGENESIS*

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Introduction

The initiating event responsible for the conversion of normal to neoplastic cells has engaged the attention of many investigators, but numerous exceptions have been encountered whenever a uniform explanation has been proposed for all cases. The existence of many mutant genes that influence the incidence of tumors, alone or in combination, is sufficiently substantiated to explain differences in hereditary susceptibility to cancer, both spontaneous and induced. However, tumors do not invariably occur in the presence of susceptibility genes, which suggests that conditions within the cell affecting critical periods when tumor genes act may also determine whether or not a cell becomes neoplastic. Therefore, examination of intracellular mechanisms and their response to intrinsic stimuli would seem a profitable method of exploration, and the methods of genetics offer one of the most accurate tools for minute, differential probing.

Euchromatic Tumor Mutants

Hereditary aggregations of cells occur in the larval stage of *Drosophila* and later regress due to the action of hormones influencing metamorphosis. They have been utilized as a prototype of inherited anomalies having characteristics in common with mammalian tumors, and the relation of euchromatin, heterochromatin, and extrinsic particles to these tumors in *Drosophila* has been examined. First, the effect of various agents on the appearance of tumors in *Drosophila* was determined[1-3] when mutation rate was known. These studies (TABLE 1) indicate that, in addition to inactive compounds, certain chemicals increase both tumor incidence and mutation rate, but others either increase mutation rate without altering tumor incidence or increase tumor incidence without a parallel increase in mutation rate. Although somatic mutation of a chromogene must be admitted as a possible causal antecedent of cancer, these results do not support the view that this is a common occurrence.

A simple loss of substance from the cell has often been suggested as a possible explanation for the neoplastic change. This would imply that known susceptibility genes that are widely distributed in the euchromatin and arise as germinal mutants are deletions. This was tested in the *tu vg bw* strain by treating males with a chemical mutagen, formaldehyde, and also with roentgen irradiation, and then determining whether reverse mutation to decreased susceptibility resulted. Although mutagenic amounts of the

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chemical were administered, no reversals occurred. On the other hand, two were recovered among 76,483 individuals treated with 2000 r and subsequently examined. The reduced incidence of tumors was inherited in subsequent generations. The original mutation from normal to completely susceptible, therefore, must have been a more complicated structural change than a deletion of euchromatin.

Table 1
Relation of Tumor Incidence to Mutation Rate

	Lethals			Tumors		
	No.	Chrom.	Percentage	No.	Total	Percentage
MCA	12	2413	0.5	126	2837	4.4*
Control	7	2366	0.5	23	1927	1.2
CH_2O	18	1174	1.5*	35	1984	1.8
Control	1	1217	0.1	30	1562	1.9
HN_2	29	781	3.7*	46	776	5.9*
Control	1	983	0.1	4	769	0.5

* $P < 0.01$.

Heterochromatin

The effect of heterochromatin on tumor incidence in *Drosophila* has been tested by determining the results of deletion of the Y chromosome and addition of extra Y chromosomes to tumor strains. A tandem X·Y chromosome was found more useful for obtaining desired genotypes than other translocations. Just as the presence of mutator genes[4] could not be correlated with increased tumor incidence, neither an increase nor a decrease in the amount of heterochromatin within the cell affected tumor incidence in a uniform manner when different strains are compared (Tables 2 and 3). These results suggest that among requisites for critical inquiry are morphologic and functional units smaller than these gross amounts of heterochromatin.

Table 2
Effect of Heterochromatin on *tu* 48*j* Tumor Incidence

	Males			Females		
	XO	XY	XYY	XX	XXY	XXYY
Tumors	62	123	27	344	267	38
Total	319	1564	873	900	2480	216
Percentage	19	8	3	38	11	18

TABLE 3
EFFECT OF HETEROCHROMATIN ON Mt^a TUMOR INCIDENCE

	Males			Females		
	XO	XY	XYY	XX	XXY	XXYY
Tumors	35	625	611	869	1782	434
Total	73	1018	985	1309	2522	584
Percentage	48	61	62	66	71	74

Genoids and Tumors

Because increasing numbers of tumors are found associated with filtrable agents, the viral hypothesis of tumorigenesis is currently ascendant. A filtrable agent that produces CO_2 sensitivity is easily transmissible in *Drosophila*,[5] and euchromatin containing genes for tumor susceptibility was introduced into cytoplasm containing the particles producing CO_2 sensitivity. Preliminary results (TABLE 4) demonstrate that such particles can reduce the incidence of tumors. This is a simple example of how the expression of tumor mutants may be altered by a viruslike particle that neither produces tumors alone nor alters the gene itself.

TABLE 4
EFFECT OF *Tr* GENOID ON *tu vg bw* TUMOR INCIDENCE

	Males			Females			Total		
	tu	Total	Percentage	*tu*	Total	Percentage	*tu*	Total	Percentage
CO_2 genoid	1555	1753	89	1466	1803	81	3022	3556	85
Control	604	608	99	546	546	100	1150	1154	100

Discussion

Apparently chromogene mutants responsible for susceptibility to the tumors investigated in *Drosophila* are structural changes other than deletions. Respective euchromatic genes for susceptibility specifically condition response to the relative dosage of heterochromatin, since results are not the same when different tumor strains are compared. Also, the necessity for testing smaller morphologic and functional units of heterochromatin for dosage effects before a very critical analysis can be made is obvious. Skepticism that any very helpful clues may emerge from studies on ploidy in general rather than from focusing attention on minute functional areas likewise seems justified.

The results with the genoid for CO_2 sensitivity illustrate the modification of action of susceptibility genes by cytoplasmic particles, the action of which s known. Similar utilization of different particles, each with specific effects, provides a promising approach for analysis of the progression of intracellular events during carcinogenesis.

Even casual examination shows that the sequential events governed by germinal mutants for susceptibility to tumors may be altered by the milieu. Therefore, whether a cancer appears may depend on alterations of genic action as well as changes in the structure of the gene or its interrelations with others. The former may represent one way in which viruses act. Since viruses, as well as genic mutations, are self propagating, their presence is an equally convenient explanation for the perpetuation of the original neoplastic conversion within the cell. In view of the similarities between the two which are frequently emphasized, the action of viruses in tumorigenesis could also be as direct as the action of genes for susceptibility. However, any number of biological systems, some of which have been reviewed[6] recently, can be used as models for possible causal mechanisms of the neoplastic process. Doubtless, all general information about the cell will be helpful in the analysis of tumorigenesis, but specific information is required for analysis of each example. The diversity of conditions known to affect carcinogenesis has thus far defied a uniform approach. Perhaps more minute scrutiny of individual cases from a mechanistic viewpoint may prove more rewarding.

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CARCINOGENIC MUTAGENS*

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Eleven years ago, in a monograph published by The New York Academy of Sciences entitled *Nutrition in Relation to Cancer*, a paper by Tatum[1] summarized the then new findings of chemical mutagenesis; that is, the mutagenic action of the sulfur and nitrogen mustards.[2-4] In the intervening decade great strides have been made in our knowledge of chemical mutagens.[5-8] Unfortunately, our understanding of the mode of action of these agents has not kept pace with their enumeration. However, Tatum's paper foreshadowed the experimental approach employed in the findings reported in this publication.

Our research has been aimed at providing answers to three main questions concerning the mutagenic activity of certain carcinogenic substances: first, "Are these substances mutagenic?"; second, "If so, what is their magnitude of activity when contrasted to other mutagens?"; third and most relevant, "If mutations are induced, what light can genetic analysis shed upon their nature?" After describing our experiments we shall attempt to evaluate their significance. Some of our earlier findings have been reported previously.[9]

While microorganisms certainly do not preempt the field as objects for mutagenic investigations, their outstanding advantage for such studies results from the lack of division into somatic and germ cells. This permits the genetic constitution of any cell to be analyzed by the standard genetic techniques of establishing a pure line or clone followed by the formal genetic procedures of crossing and progeny analysis. Furthermore, the use of microorganisms circumvents the difficulties of establishing effective and known concentrations in germ plasm, a problem that has plagued other investigations in this field. Of course, not all microorganisms possess these attributes to an equal degree. The system selected to evaluate the carcinogens in the work here reported was the induction of nutritional (biochemical) and morphological (visible) mutants in the fungus *Neurospora crassa*. This system has been referred to by Giles as the induction of forward mutations, as opposed to back mutations.[10] Nothing evolutionary is implied in the term. The significance here is that the forward system permits the detection of mutations of a very large number of genetic loci simultaneously, whereas the back mutation technique permits the detection of mutations at one or, at most, only a few genetic loci. The use of drug- or virus-resistant mutants has the same disadvantage of measuring mutations of only a few genes. The disadvantage of this forward system is that the labor involved

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in detecting mutant strains is many times greater than in the back mutation techniques.

The first experiments were carried out according to the original technique devised by Beadle and Tatum.[11] Conidia of one mating type were treated with the substance under test and immediately were used to fertilize sexual bodies of the opposite mating type. The resulting ascospores, the products of meiosis, were isolated, and the cultures derived from them were examined for the presence of nutritional and morphological mutant strains. Using the water-soluble derivative of 20-methylcholanthrene, sodium 6,12b-endo-20-methylcholanthrene succinate prepared according to the method of Bachmann and Kloetzel[12] at *p*H 6.0 at a concentration of 0.1 per cent, and treating conidia for periods up to 80 min., 4 biochemical mutants were found among 1700 isolates. This is a frequency of approximately 0.24 per cent,* a value considered significantly above the spontaneous rate, although no accurate spontaneous rate had then been ascertained. For comparison, a frequency of about 2 or 3 per cent biochemical mutants was obtained from material similarly treated with X rays, ultraviolet irradiation, or nitrogen mustard. These preliminary results were considered indicative of a positive mutagenic action. A more detailed investigation was undertaken.

Methods

In investigations of chemical mutagenesis some criterion of penetration of the agent under test into the cells is essential. Death of a large fraction of the cells is commonly employed, since most mutagens are toxic agents—for example, the nitrogen and sulfur mustards. In practice, the higher the kill, the greater the mutation rate; that is, the percentage of mutants among the survivors. There are a few noted exceptions that need not be considered here. In the evaluation of the carcinogens, killing was not observed except in certain experiments with the endosuccinate derivative of methylcholanthrene, in which the killing effect may have been indirect. Considering the extremely low water solubility of the carcinogenic hydrocarbons (1 part in 10^8 or less), and the fact that *Neurospora* grows in an aqueous medium, the problem became even more relevant. Therefore it was necessary to employ a different criterion as evidence that the compound actually penetrated the cell (mycelium).

For the penetration studies to be described, 20-methylcholanthrene at a concentration of 0.025 per cent was employed. As a qualitative test for penetration, mycelia grown in methylcholanthrene were washed and examined under the microscope at 1000 diameters for fluorescence under ultraviolet light.† No change in the optics of the microscope was found necessary. A quantitative measure of penetration was provided by refluxing washed methylcholanthrene-grown mycelium in ethanol and determining the amount of methylcholanthrene extracted in the ethanol. The absorption at 295.5 mμ was found to be proportional to concentration and accurate within 1 mμ for quantities of less than 10 mμ.

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* The actual frequency would be twice this value since only one half of the isolates carry the allele at any locus derived from the treated parent.

† A mercury-vapor arc with a Corning No. 5840 filter (transmission 310 to 395 mμ) was employed as the light source.

Three methods of solubilizing the test substances were evaluated: (1) a solution of the carcinogen in ethanol was added to culture medium, and the resulting colloidal suspension was immediately inoculated with *Neurospora* i (2) the water-soluble endosuccinate derivative described above was prepared and added to culture media; and (3) the carcinogen was dissolved in polyoxyethylene sorbitan monooleate (Tween 80),* and this in turn was dissolved in the culture media. Based on the two criteria just described the Tween 80-methylcholanthrene preparation was completely penetrable to the cells. Abundant fluorescence was observed in individual hyphae of *Neurospora* grown in the Tween-methylcholanthrene. Such mycelia, when frozen and crushed, freed many highly fluorescent particles from ruptured cells. Mycelia grown in the basic media with only Tween added failed to fluoresce at these wave lengths. Mycelia grown for 1 week in the culture media with 10 per cent Tween plus 0.025 per cent 20-methylcholanthrene added were found to be in equilibrium with the culture medium with respect to the methylcholanthrene concentration; that is, the concentration in the alcohol extract of the mycelium was found to be equivalent to a mycelial concentration of 0.025 per cent when calculated on the basis of the wet weight of the mycelium. On the basis of these results all further experiments were carried out by growing mycelium for a period of 1 week in medium containing 10 per cent Tween as a solubilizing agent for the carcinogen. Other experiments established that the azo dyes also penetrated the cells. It should be borne in mind that, contrary to the usual method of evaluating mutagens, these experiments were performed at nonkilling concentrations of the agents under tests as mutagens.

To render the evaluation of the carcinogenic substances more precise, two modifications of the experimental procedure of Beadle and Tatum[11] were introduced. The first of these modifications circumvents the necessity of resorting to the sexual stage in order to isolate pure strains; that is, strains containing only one nuclear type. In this way recombination as a source of variation is eliminated. When *Neurospora* multinucleate conidia are treated, recourse to a sexual cross followed by ascospore isolations constitutes the only sure method of isolating pure clonal lines from mutagen-treated material. During the course of these investigations a strain of *Neurospora* was developed that produces almost exclusively uninucleate conidia (microconidial of Barratt and Garnjobst[13]). By plating microconidia into a medium that restricts the spreading growth form,[14] *Neurospora* may be treated as an asexual form, and clones may be isolated by the standard microbiological techniques for isolating pure cultures. In order to minimize the biological variation from experiment to experiment, a second modification was introduced. The microconidial strain selected was made homocaryotic by the isolation of a colony derived from a single uninucleate microconidium. This colony in turn was grown to sporulation, and these spores were immediately lyophilized in sufficient replication to provide a new culture from lyophil for each experiment.

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* Manufactured by Atlas Powder Company, Wilmington, Del.

In each of the experiments evaluating a carcinogen as a mutagen, the following experimental procedure was used (FIGURE 1):

(A) A lyophil tube containing conidia of the purified strain was opened and the contents were used to inoculate an agar slant.

(B) The culture was grown 4 to 5 days at 25° C. until conidiation occurred, and a suspension of microconidia was prepared. The experimental flasks that contained synthetic culture medium[11] and a carcinogen in Tween, as well as a control flask containing Tween and culture medium only, were inoculated with the suspension. Approximately 10^7 conidia were added to 40 ml. of medium in the culture flask.

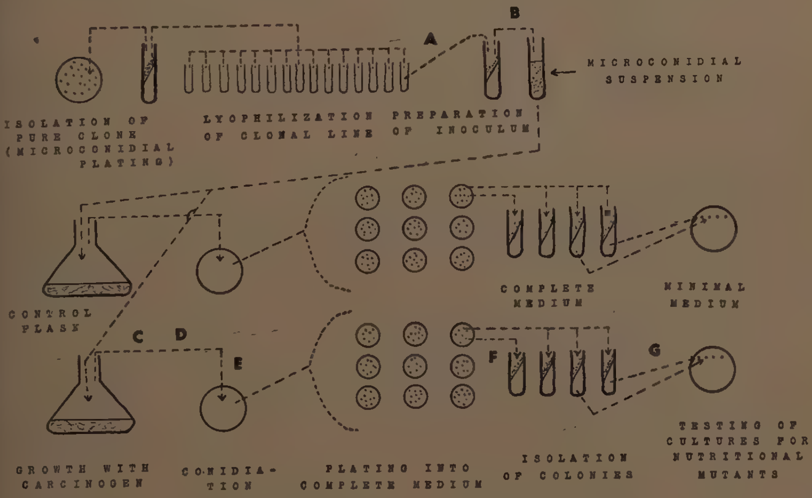

FIGURE 1. Experimental procedure for the evaluation of carcinogens as mutagens. The letters correspond to procedural details described in the text.

(C) After 7 days' growth at 25° C. the mycelium in each culture flask was filtered free from the medium with the aid of sterile filter paper in a sterile Büchner funnel, and the mycelium was washed.

(D) The mycelium and filter paper were transferred aseptically to a Petri dish containing complete agar medium[11] and were incubated for 3 days at 25° C. to induce abundant microconidiation.

(E) The conidia were scraped from the surface of the mycelial mat, suspended in water, filtered through cotton to remove mycelial fragments, and plated onto glycerol-sorbose complete agar plates[14] at a density calculated to give rise to about 100 colonies per plate.

(F) The plates were incubated for 3 days at 30° C. to permit colony formation. One thousand colonies from each treatment were isolated to individual agar slants of complete medium and incubated at 25° C. for 5 to 6 days.

(G) Each culture was scored for deviation from the purified microconidia strain (morphology); each culture was tested for a nutritional mutation by transferring onto minimal sorbose agar plates in a pattern of 25 per plate and was scored for growth after 48 hours at 30° C.

(H) Cultures failing to grow on the minimal agar were tested for the exac nutritional requirement according to the standard procedure of Beadle an Tatum.[11] All mutants obtained from a single experiment that had the same nutritional requirement were tested for allelism by a heterocaryon test Similar heterocaryon tests were performed for the morphologic mutants In any questionable cases the mutant strain was crossed to the wild type the opposite mating type was isolated, and a direct cross was made with the strain in question. Recovery of wild type isolates from the cross was take as evidence for nonallelism.

Results

Three carcinogenic hydrocarbons, 20-methylcholanthracene, 9,10-di-methyl-1,2-benzanthracene, and 1:2,5:6-dibenzanthracene; two azo dyes *m*′methyl-*p*-dimethylaminoazobenzene and 4′amino-2,3-azotoluene; one stil-bene, 4-dimethylaminostilbene; and 2-acetylaminofluorene were evaluated TABLE 1 summarizes the biochemical or nutritional mutants obtained fro experiments performed in the manner described above. TABLE 2 summarize the total mutants obtained in the treated material; that is, nutritional plu visible.

TABLE 1
CARCINOGEN MUTAGENESIS IN *Neurospora*
Biochemical Mutants Obtained from Carcinogen Treatments

Mutagen and concentration	Number of experiments	Number of isolates tested	Number of mutants	Per cent mutants
Control	11	9658	3	0.03
20-Methylcholanthrene (0.025%)	5	6347	11	0.17
9,10-Dimethyl-1,2-benzanthracene (0.025%)	3	2501	3	0.12
1:2,5:6-Dibenzanthracene (0.025%)	2	1500	3	0.20
m′-Methyl-*p*-dimethylaminoazobenzene (0.050%)	3	2615	3	0.11
4′-Amino-2,3-azotoluene (0.1%)	4	3713	0	0.00
4-Dimethylaminostilbene (0.025%)	3	2771	2	0.07
2-Acetylaminofluorene (0.1%)	3	2775	1	0.04

Before any significance concerning mutagenicity is attached to these find ings, the possibility must be excluded that these mutants represent selectio by the carcinogen from possible pre-existing mutants in the population o conidia used as inoculum. Since growth occurred for a period of 7 day while the mutagen was in contact with the mycelium, ample time existe for selective action. To test this possibility as a factor, a reconstructio

TABLE 2

CARCINOGEN MUTAGENESIS IN *Neurospora*

Total Mutants Obtained (Biochemical and Morphological) from Carcinogen Treatments

Mutagen and concentration	Number of experiments	Number of isolates tested	Number of mutants	Per cent mutants
Control	14	12431	33	0.27
20-Methylcholanthrene (0.025%)	7	7107	47	0.66
9,10-Dimethyl-1,2-benzanthracene (0.025%)	3	2501	15	0.60
1:2,5:6-Dibenzanthracene (0.025%)	2	1500	9	0.60
m′-Methyl-*p*-dimethylaminoazobenzene (0.050%)	3	2615	11	0.42
4′-Amino-2,3-azotoluene (0.1%)	4	3713	16	0.43
4-Dimethylaminostilbene (0.025%)	3	2771	6	0.22
2-Acetylaminofluorene (0.1%)	3	2775	8	0.29

experiment was carried out. Conidia from 4 mutant strains (representatives from both the morphological and biochemical classes obtained in these studies) were mixed in a predetermined ratio with wild type conidia and used as inoculum for an experiment conducted exactly as described above. If the carcinogen were not inducing mutations but acting as a selective agent, then a larger proportion of mutants would be recovered among isolates made after growth in the presence of the carcinogen than were originally present in the inoculating mixture. From the data presented in TABLE 3 it is apparent that none of the 4 mutant strains was selected for; in fact, they were all selected against, as in each case a much smaller percentage of the mutant was recovered at the end than was present in the inoculum. This permits the conclusion that the mutants obtained in the carcinogen treatments in

TABLE 3

RECONSTRUCTION EXPERIMENT: TEST FOR SELECTIVE ACTION OF 20-METHYLCHOLANTHRENE ON A MIXTURE OF WILD TYPE AND MUTANT MICROCONIDIA

Strain	Per cent in original mixture	Number in 1360 isolates	
		Expected	Obtained
Wild type	90.5	1232	1351
Adenine (#S1644)	4.2	56	3
Histidine (#S2570)	2.2	30	3
Cytidylic acid (#S1672)	2.7	37	2
Colonial (#S1609)	0.4	5	1
Total	100.0	1360	1360

excess of the controls were induced by the treatment. Based on the nutritional mutants obtained, all 3 of the carcinogenic hydrocarbons and 1 of th azo dyes are mutagenic, giving mutation frequencies 4 to 6 times the contro frequency. The stilbene and aminofluorene tested were not detectabl mutagenic. Based upon the total mutants obtained, all 3 hydrocarbon and both azo dyes are mutagenic, giving mutation frequencies of 2 to 2. times the control.

Table 4 lists the specific nutritional requirement for each of the nutritiona mutants obtained. The most frequent types of nutritional mutants induce with any mutagen in *Neurospora* are methionine (or methionine-cystine and adenine.[4] It is of interest to note that, of 3 spontaneous nutritiona mutants that occurred in the control samples, 2 fall into these classes. Of the 2 tryptophan mutant strains obtained, 1 in the material treated with dimethylbenzanthracene and the other with dimethylaminoazobenzene, both are uncommon types of tryptophan mutants. All but 1 of the mutant types isolated have also been obtained with other mutagens, but only the adenine-, methionine-, and proline-requiring strains have been obtained spontaneously. The strain requiring choline and *p*-aminobenzoic acid has not been reported before.

TABLE 4

CARCINOGEN MUTAGENESIS IN *Neurospora*

Requirements of Biochemical Mutant Strains Obtained from Treated Material

Mutagen and concentration	Number of mutants	Requirements
Control	3	Adenine, methionine, proline
20-Methylcholanthrene (0.025%)	11	Proline, adenine, cytidylic acid (2), methionine, histidine, lysine, amino acid, amination, tyrosine, threonine
9,10-Dimethyl-1,2-benzanthracene (0.025%)	3	Adenine (2), tryptophan
1:2,5:6-Dibenzanthracene (0.025%)	3	Yeast nucleic acid, methionine-cysteine, amino acid
m′-Methyl-*p*-dimethylaminoazobenzene (0.050%)	3	Lysine, tryptophan, adenine
4′-Amino-2,3-azotoluene (0.1%)	0	—
4-Dimethylaminostilbene (0.025%)	2	Unknown (died), methionine
2-Acetylaminofluorene (0.1%)	1	Pab + choline

In order to learn more about the nature of the mutation in these strains, formal genetic analyses have been carried out on most of the biochemica strains. Approximately 1:1 ratios of the mutant and wild type characteristics have resulted from crosses of each of these strains with the wild type. Furthermore, those strains analyzed by tetrad analysis regularly give rise to asci containing all 4 viable meiotic products. These data provide reasonably conclusive evidence that these mutant strains do not contain gros chromosomal aberrations. Direct cytological evidence for aberrations is very difficult to obtain in *Neurospora*.

Having established that no gross aberrations were present in these carcinogen-induced mutant strains, it was of interest to ascertain whether these strains were capable of reverting to prototrophy. The frequency of reversion to prototrophy of 3 of the 20-methylcholanthrene-induced biochemical mutants was investigated. For the reversion studies, ultraviolet radiation from a Westinghouse Sterilamp was employed. The 3 strains selected* were an adenine-requiring, a proline-requiring, and a cytidylic acid-requiring strain. As a control, an inositol-requiring strain was used that was ultraviolet-induced and that had been shown to revert.[14] The 4 strains were irradiated and plated onto a minimal sorbose agar medium. The effect of the irradiation on viability was determined by plating an aliquot of the conidia onto a complete medium. On the minimal medium the only conidia capable of growth into colonies would be those independent of their original requirement, and thus prototrophic. As shown in TABLE 5, all 3 of the carcinogen-induced strains investigated are capable of reverting to prototrophy under irradiation with ultraviolet light.

TABLE 5

REVERSIONS INDUCED BY ULTRAVIOLET IRRADIATION IN 3 CARCINOGEN-INDUCED MUTANTS AND 1 ULTRAVIOLET-INDUCED MUTANT

Strain and requirement	No. of expts.	Per cent survivors (mean)	No. survivors ($\times 10^8$)	No. of reversions obtained	Reversions per 10^6 survivors
S1644 adenine (C)	7*	34.8	1.9	30	0.15
S1639 proline (C)	2	4.0	0.3	8	0.50
S1672 cytidylic acid (C)	3*	43.5	4.2	5	0.012
37401 inositol (UV)	5*	34.8	0.7	84	1.15

Symbols: C = carcinogen-induced strain; UV = ultraviolet-induced strain.

* Several of the experiments consisted of several irradiation doses, which actually could be considered separate experiments.

Prototrophs may arise either by back mutation at the original locus or by suppressor mutation at another locus. These 2 alternatives can be distinguished by analyzing the progeny obtained in a cross of the revertant to a wild type. Six reverted strains representing 2 isolates from each original strain were crossed to the wild type, and the progeny were isolated and analyzed.† In a cross between a reverted culture (phenotypically wild type) and a normal strain (genotypically wild type) only wild type progeny will be obtained if a true back mutation has occurred; while if a suppressor mutation has occurred, segregation of the suppressor locus and the original mutated locus will give rise to mutant progeny. None of the isolates from any of the 6 crosses of the revertants to wild type was a mutant. The con clusion is that the reversion in each of the 6 cases analyzed genetically wa due to a true back mutation at the locus originally mutated by the carcinoge

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* These strains were selected on the basis of inability to form microcolonies on minimal medium.

† More than 300 isolates were made from crosses of each reverted strain.

From these investigations of the genetic stability of 3 carcinogen-induce biochemical mutants, it appears that the alterations of the genetic materia fall into the class of gene mutations.

Discussion

Regarding the merits of detecting forward mutation versus detecting bac mutation, clearly the back mutation technique, so elegantly employed b many investigators, yields more quantitative data. The limitation lies i the few loci in which mutations can be detected in a single experiment Many investigators have reported marked differences in the mutageni action of different agents when determined by their relative action on 2 loc Novick[8] observed a difference between the mutagenic action of purine deriva tives and ultraviolet light on mutation to phage T5 and T6 resistance Giles[15] has observed the same phenomenon when studying back mutation in *Neurospora* at two loci concerned with nutritional requirements. Also i *Neurospora*, Kolmark[16] observed a marked difference between the mutageni action of ultraviolet light and 1:2,3:4-diepoxybutane on the frequency o reversion at an adenine and an inositol locus. It should be noted tha Jensen *et al.*[17] found methylcholanthrene inactive in inducing back mutation at the one locus studied in *Neurospora*. Based on studies of the action o three mutagens on several loci in *Escherichia coli*, Demerec[6] concludes tha different genes react differently to similar treatment with a given mutagen, phenomenon he terms "mutagen specificity." In the light of these an similar findings of mutagen specificity, the forward mutation techniqu seems to have provided suitable material for this investigation.

In attempting to evaluate the significance of these findings one is impresse by the low frequency of mutations obtained. These investigations and thos reported by Novick using the chemostat are among the very few example of mutagens used under nonkilling conditions. Novick[8] observed increase in frequency up to tenfold with caffeine and other purine derivatives. Th chemostat of Novick and Szilard[18] provides an environment in which a large constant, growing population of bacteria can be maintained for long period of time. It permits a very accurate measure of mutation at a few geneti loci, but is not suited to the simultaneous measure of mutation at many loci The more general questions are concerned with what constitutes an accurat measure of the mutagenic activity of a compound and what common denominator should be used in contrasting mutagens. Clearly, to measure mutagenic activity of a series of compounds at concentrations of the agents that give equal kill, say at the 5 or 1 per cent survival level, is not a useful yardstick for evaluating nontoxic substances. Parenthetically, it is not certai that the selection of equal killing levels is valid even for comparing mutagen having a toxic effect; these questions cannot be answered until we know muc more about the mode of action of mutagens (Auerbach[5]). The significanc of the results described in this paper lies, not in the frequency of mutagenesi bserved, but in the positive mutagenic activities. One would not necessarily expect a high degree of correlation between mutation *frequency* and carcinogenicity. The conditions, period of contact, effective concentration, and probably gene mutability are so vastly different when carcinogens are applied to mammalian tissues that they negate any attempts at quantitative comparisons.

Even valid qualitative comparisons are difficult between results of experiments on mutagenesis in microorganisms or other lower forms and on carcinogenesis in mammals. Insofar as such comparisons are possible, it would seem that only positive correlations are significant. The demonstration of even weak mutagenic activity of a carcinogen in any organism would seem to constitute good grounds for the assumption that it is capable of inducing genetic changes in mammalian cells, since genic material is basically similar in all living organisms. Negative tests for mutability are subject to the same question as any negative results, and may be attributable to basic differences in cell metabolism. For example, there is a considerable body of evidence that metabolic products of the carcinogens are the biologically active molecules. Since it is unlikely that cells of *Neurospora* and mammalian tissues would metabolize carcinogens identically, this difference may lead to different qualitative as well as quantitative effects of carcinogens in the two cases, as in the apparent mutagenic inactivity of 4-dimethylaminostilbene and 2-acetylaminofluorene in *Neurospora*.

Another important factor in evaluating these results is the role of selection on the mutations produced. Again one would expect a greatly different situation in microorganisms than in mammalian tissues. Confining consideration to *Neurospora* for the moment, the reconstruction experiment reported in this paper suggests that, under the conditions of our experiments, nuclei containing mutations for both nutritional and visible characteristics were strongly selected against during the growth of the culture. One might infer that the actual mutation frequency was much higher. Our assumption was that, because of the nontoxicity of these agents, contact for a long period of time between the carcinogen and mycelium was desirable in order to permit maximum mutagenic action. This may have been an erroneous assumption. Even if it were possible to design experiments that would shed more light on these two factors—the optimum period of contact for mutagenic action and selection against the induced mutants—the results would add little to our understanding of the action of these agents in mammalian cells and tissues. Unfortunately, only the most rudimentary knowledge exists on the selective forces operating on a hypothetical mutant cell of the type that would give rise to malignancy in mammalian tissues. They are undoubtedly very different from those existing in the experiments just described. Parenthetically, if mutation is responsible for carcinogenesis, understanding of just these complex selective factors would seem basic to practical therapy and control of cancer. The recent dramatic advances in tissue culture of mammalian cells now make studies of this sort practicable.

Haldane[19] long ago pointed out that the mutation hypothesis of cancer is experimentally untestable, since a formal genetic analysis of mammalian somatic cells is impossible. Conversely, the objection is raised that organisms such as *Neurospora*, in which division into germinal and somatic cell does not exist, do not possess a degree of cellular organization that would make it possible to detect a "malignant cell type." Indeed, the data in thi paper establish only that certain carcinogens are weak mutagens and tha the mutations recovered are gene mutations. Any significance is base upon the extrapolation that assumes that mutation is a common property of all genetic material and that cell-to-cell organization in a mammalian organism is ultimately gene controlled. Certainly the mechanism of gen control of cell-to-cell organization remains to be established; that is, how fa a differentiated cell is controlled by its genetic make-up and how far by it being differentiated, remains an open question. This has been eloquentl pointed up by Schultz elsewhere in this monograph.

Considerations such as those briefly discussed here obviously make impossible a definitive conclusion as to the role of mutation in carcinogenesis We feel, however, that the mutagenic activities of the carcinogens here reported, and other similar findings, are at least not inconsistent with the hypothesis that somatic mutation is involved in carcinogenesis. Unfortunately, it is equally clear that experiments with any nonmammalian organisms can never provide more than circumstantial evidence on this question A final answer can be reached only through studies on the biological and biochemical behavior of human cells, and on the correlation of these propertie with genetic constitution.

To date, genetic analysis of malignant cells has not been accomplished We have now come full cycle in Haldane's argument, but certainly the future for genetic analysis of somatic cells does not appear too discouraging The parasexual cycle elucidated by Pontecorvo[20] and his associates establishes the fact that, at least in completely asexual forms of fungi, a mechanism for genetic analysis is available. Asexual nuclear fusion, recombinatio via somatic crossing over, and the return to haploidy via aneuploids are al parts of the parasexual cycle. The fusion of cells in sarcoma 180 in the ascitic form observed by Law[21] may, we hope, be a first step in genetic analysis of somatic cells of higher organisms. The chromosomal behavior in cell of mouse tissue cultures reported by Levan in this monograph may provid material for genetic analysis of somatic cells. The genetic phenomena o transduction and transformation observed in certain bacteria may have thei counterpart in mammalian cells (Lederberg[22]). Thus, the time may not b too far distant when a rigorous testing of the hypothesis of the mutationa origin of cancer will become feasible.

Summary

Eight carcinogenic substances were evaluated for mutagenic action a measured by the induction of biochemical and morphological mutant strain in the fungus *Neurospora crassa*. The technique employed consisted c growing a microconidial strain in the presence of the carcinogen, transferrin the mycelium to a medium on which conidiation would occur, plating th conidia, and isolating and testing colonies derived from single microconidia

Based on the biochemical mutants recovered, it is concluded that 20-methylcholanthrene, 9,10-dimethyl-1,2-benzanthrene, 1:2,5:6-dibenzanthrene, and *m*′-methyl-*p*-dimethylaminoazobenzene are weakly mutagenic. Based upon the total mutants recovered, biochemical plus morphological, in addition to the above compounds 4′-amino-2,3-azotoluene is weakly mutagenic. 4-Dimethylaminostilbene and 2-acetylaminofluorene were found not to be detectably mutagenic.

Formal genetic analysis was carried out on most of the biochemical mutant strains. No evidence was found that any of the strains contained gross chromosomal aberrations. Three of the 20-methylcholanthrene-induced biochemical mutant strains were examined for reversion to prototrophy, and all were found to revert. Genetic analysis of two revertants of each strain established the fact that the reversion was due to back mutation at the original locus.

The implications of these findings are discussed.

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MUTATIONS OF VIRUSES IN RELATION TO NORMAL AND ABNORMAL CELL FUNCTION*

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Bacteriophage Mutations and the Genetics of Bacteriophage

Variation is observed in viruses of all groups and can affect any of their detectable properties, including host range and pathogenicity. The mechanism of mutation and the effects of mutation on virus growth and function at the cellular level have been studied only on bacteriophages. With plant viruses, on the other hand, some information exists on the effects of genetic variation on the chemical composition of the virus protein (Knight, 1957).

The peculiar opportunities of the bacteriophage-bacterium system, and the identification of the phage genome with desoxyribonucleic acid (Hershey and Chase, 1952), make possible quantitative studies of the process of phage mutation itself and of its relation to phage chemistry and replication; of the organization of the mutable elements in the phage genome, through the analysis of genetic recombination; and of the function of the mutable elements, by the analysis of the functional blocks and changes in phage functions brought about by mutation.

Thus, we know that spontaneous phage mutations occur by random events that take place during exponential replication of phage genomes (Luria, 1951); that the mutable elements behave in "crosses" as stable, recombinable units linked in a linear array (Hershey and Rotman, 1948; Benzer, 1957); that mutations can result from incorporation of pyrimidine analogues into the phage desoxyribonucleic acid (DNA) during its biosynthesis (Litman and Pardee, 1956); and that the quantal element of mutation and recombination comprises at most a few nucleotides, more probably a single one, whereas the quantal element of genetic function or "cistron" extends over hundreds or thousand of nucleotides (Benzer, 1957).

There are some phage mutations that express themselves, not (or not only) as alterations in the properties and growth of the phage itself, but also in the properties and functions of the bacterial host cells. These mutations provide useful models for our consideration of the cancer cell and the role of viruses in normal and abnormal developmental processes, and constitute the subject matter of this paper.

Genetic Functions of Bacteriophage

Whenever virus infection does not lead to immediate cell destruction it becomes possible to detect changes in cellular functions that appear to be controlled by the virus acting as a genetic determinant. The tumor viruses are the most appropriate example of a viral control over cellular morphogenesis, that is, over the cellular response to the regulatory activity of the environment.

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Even in rapidly destructive viral diseases, such as the lytic infection of bacteria with phage, we can interpret individual events in viral biosynthesis and cellular dysfunction as due to specific genetic functions of the virus that can be altered by mutation. With phage, these include: (1) control over reproduction of viral DNA; (2) control over production of enzymes specifically needed for synthesis of DNA constituents (Barner and Cohen, 1954; Flaks and Cohen, 1957); (3) suppression of synthesis of certain cellular enzymes (Cohen, 1949); (4) control over the synthesis of viral proteins, which behave as gene products synthesized at large in the cell and used in the final building of mature phage particles (Streisinger, 1956); (5) control over lytic enzymes, which may play a role in cellular lysis (Huppert and Panijel, 1957); and (6) control over enzymes that can destroy slime layers around the cells (Adams and Park, 1956).

It is, however, in the persistent type of infection (that is, in lysogenic bacteria) that the genetic control by phage over what appear to be purely cellular properties is most evident. In lysogenic bacteria the phage is present as "prophage" (Lwoff, 1953) in every cell; it manifests itself by the occasional production of mature phage, liberated by lysis of a certain proportion of the cells. Besides the production of phage, the prophage controls a number of other cellular characters. These include a whole range of properties, from immunity to lysis by phages related or unrelated to the prophage (Bertani, 1953), to ability to produce toxin (in *Corynebacterium diphtheriae*, Freeman, 1951), to changes in the antigenic composition of the bacterial surface (Iseki and Sakai, 1953).

These genetic functions express themselves in what have been termed "conversions," that is, in persistent differences between lysogenic and nonlysogenic cells. Since prophages, at least in cases analyzed in *Escherichia coli*, appear to be located at specific sites in the bacterial chromosome (Lederberg and Lederberg, 1953; Bertani, 1958), conversions might express functions specifically exerted by prophages acting from their chromosomal location. Our recent analysis of the control of conversion of *Salmonella* antigens by a phage (Uetake *et al.*, 1958), illustrated in FIGURE 1, shows, however that the new property—formation of somatic antigen 15 and disappearance of antigen 10—appears within minutes after infection, even in cells destined to lysis. The antigenic change reaches nearly complete expression even in infected cells that survive and, by segregation, lose the phage within a few generations. The phage genome, however, is not simply an activator of a latent potentiality of the cells. In the cells that lose the phage, the new antigen is diluted away, and the cells resume synthesis of the original antigen To paraphrase Rous' dictum (1943) about tumor viruses, the phage is the persistent and actuating cause of the conversion in cellular functions. I shal return below to this example to illustrate the role of mutation in changing virus-controlled cellular properties to what appear to be persistent, nonvira properties.

It may be worthwhile pointing out here the suggestive analogy between irus-controlled changes that make bacterial cells agglutinable or unaggluti-able by specific antisera, and the changes involved in cancerization, which hake cells unresponsive or over-responsive to growth-restraining or growth-romoting substances in their environment.

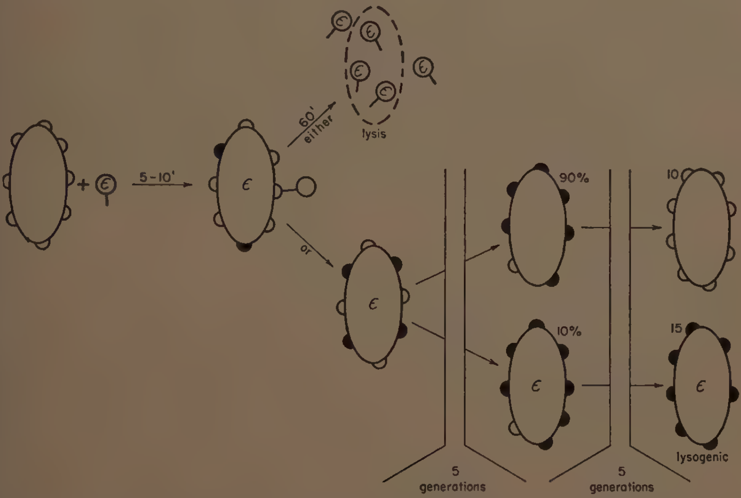

FIGURE 1. Antigenic changes controlled by phage ϵ^{15} in *Salmonella*. The oval sym-ols represent bacterial cells. The empty areas on the bacterial surface represent ntigen 10; the full areas represent antigen 15. Phage particles are represented by the ircles with attached tails. The phage genetic material is identified by the letter ε. Segre-ation of lysogenic and nonlysogenic cells (10 per cent and 90 per cent, respectively) occurs the progeny of the infected cells that survive (Uetake *et al.*, 1958).

Mutations Affecting the Relation of Phage to Cellular Functions

Mutations from temperance to virulence. A phage that can lysogenize the acteria it infects is called temperate; a phage that cannot do so is called irulent. Mutation from temperance to virulence has long been known. genetic analysis of such mutations (Levine, 1957; Kaiser, 1957) has revealed hat a number of functions are required for lysogenization, each function eing controlled by a specific "cistron" in the phage genome and subject to enetic blocks by mutations at any one site within that cistron. In mixed fection, mutants blocked in different functions can cooperate in producing ysogeny. Some of the mutations, however, also suppress the ability of the hage to become a stable prophage; these mutant phages may persist in fected cells for a few generations, but the cells that carry them will soon e destroyed.

Thus, the mutations to virulence eliminate the ability of a virus to become persistent genetic determinant within a persistent cell lineage. They onfirm the surmise of a continuous transition and intimate relationship etween the destructive and the proliferation-inducing types of animal viruses

(Duran-Reynals and Shrigley, 1945) and specify the details of the mutationa process by which one type may arise from the other.

Mutations of prophages to defectiveness. Occasionally a lysogenic ba terium gives rise to mutants that are defective in lysogeny (Jacob an Wollman, 1956). Production of phage is absent, but other prophage-con trolled properties, such as immunity and susceptibility to lysis by exposur to "inducing" agents (Lwoff, 1953), persist. The defect is due to a mutatio in the prophage, and can be repaired by back mutation. The mutationa site can be located in the linkage map of the phage (Appleyard, 1954).

The importance of these genetic defects of prophage is that they suppre the viral aspect without eliminating the other genetic functions of the pro phage. This is illustrated in FIGURE 2, based on unpublished observatio by H. Uetake on *Salmonella* antigens. The defective bacterial mutants d not produce any phage, but retain the phage-controlled antigen 15. Tha the defect is in the prophage itself is indicated here by the fact that when a additional mutation (presumably a loss of the defective prophage) remove all prophage-controlled functions, relysogenization with normal phag restores the normal lysogenic condition.

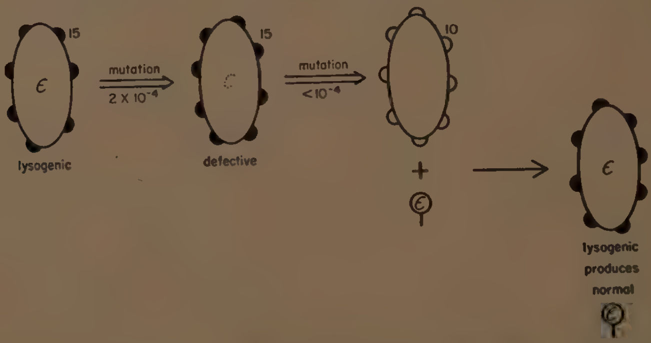

FIGURE 2. Mutations to defectiveness and to loss of prophage in *Salmonella* lysogen for phage ϵ^{15}. The broken letter indicates the defective prophage (H. Uetake, unpu lished data).

Thus, a cellular character such as the specific surface antigen, which initiated by virus infection and maintained by a persistent provirus, remain unchanged when the provirus become operationally indistinguishable fro any "nonprovirus" fragment of host genome. The sequence virus → pr virus → defective provirus illustrates the transformation of infectio heredity into stable cellular heredity. The reverse mutation, from defecti provirus to normal provirus, illustrates a mechanism by which cellular cha acters may acquire transmissibility by viral infection.

Special transduction by phage λ. The final instance to be discuss (FIGURE 3) is the genetic change that gives rise to "high-frequency tran duction" by phage λ (Morse *et al.*, 1956; Campbell, 1957; Arber *et al.*, 195 In the lysogenic bacteria the prophage λ is located near the Gal genes co trolling galactose utilization. These bacteria produce, among normal pha

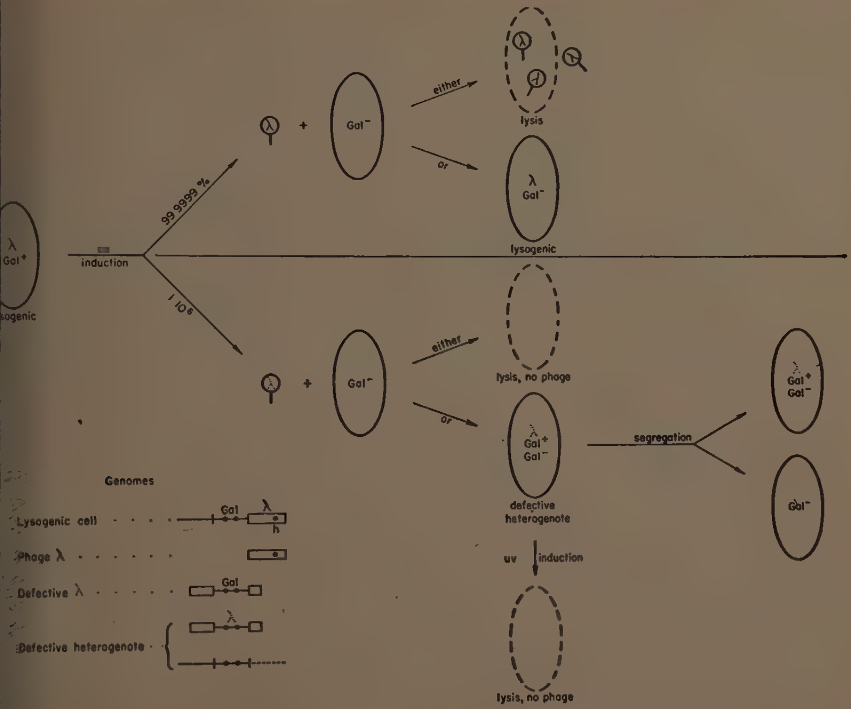

FIGURE 3. The transduction of Gal locus by defective phage λ (based on data by rber *et al.*, 1957). The genetic material of the defective phage can persist in unstable rm in the progeny of infected bacteria. These bacteria are detected as heterogenotes for ie Gal characters. The insert represents one interpretation of the genetic constitution phages and bacteria, based also on experiments on phage recombination.

articles, a small proportion of Gal-transducing particles that can transduce ecifically to a recipient cell the Gal genes of the donor cell. These phage articles, however, are defective. They lack the ability to initiate phage roduction and to become persistent, stable prophages; the recipient cells arry the tranduced Gal genes, apparently coupled with the defective phage ement, in a "heterogenote" state. The defective phages lack some of the hage gene loci, and the Gal region may actually replace the missing phage enes.

This example illustrates another type of mutational change by which a agment of cell genome may become subject to viral transmission. Here ie mutation appears to involve a chromosomal rearrangement between prophage element and a neighboring genetic element of the bacterial iromosome.

Conclusion

Phenomena of transformation (Hotchkiss, 1956) and transduction (Zinder, 955) have indicated that all elements of the bacterial chromosome are otentially transferable from cell to cell if they happen to gain access to a receptive cell, either through artificial chemical manipulations by an exper menter, or naturally, through accidental inclusion into a viral vector particl Infectionlike transfers of genetic elements of animal and plant cells, whethe chromosomal genes or "plasmids," have been inferred from evidence of man kinds (Lederberg, 1952).

Studies on bacteriophage have led us to consider these viruses as fragments of hereditary material that are not only transferable but also endowe with the genetic ability to control their own maturation into virus particle that is, to determine the synthesis of a specific apparatus designed for the own transmission to other cells. The degree of resemblance between vira and nonviral fragments of the bacterial genome cannot yet be evaluate fully; for example, we do not know whether a prophage acquires its chromosomal location by attaching itself sidewise to an otherwise linear chromosoma sequence, by attaching to the chromosome as one of many similar sid chains, or by inserting itself within the linear chromosome, possibly b recombination with a homologous region lacking the gene alleles required fo viral development.

The examples discussed in this paper illustrate known instances of mutational changes from genetic elements that behave as "pure viruses," suc as virulent phages, to genetic elements that behave as nonviral determinant of heredity. These mutational transitions, and the similarity of gene actio exerted by viruses in destructive and in persistent types of infection, indicat that gene control and virus control over cellular functions are two aspect of the same genetic mechanism. This concept removes any *a priori* incompatibility between a viral and a genetic theory of cancer etiology. It reduce the interpretation of virus-induced cancer to that of the control of cellula development and differentiation by genetic elements capable, either intrinsically or by association with other specialized genetic units, of assuming a infectively transmissible form.

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GENETIC ASPECTS OF HORMONAL INFLUENCES ON CANCE

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This is a particularly difficult topic to discuss because so much of wh is known about the subject is in terms of species differences or strain diffe ences, little having been done on the genetic analysis of these qualitati differences. The strain differences are often quantitative; they appear stages of ontogeny that are late or that are conditioned during prolong periods of study. The genotype predisposing to—or, we might assume f the time being, responsible in part for—tumors related to endocrine di turbances usually becomes manifest late in the life of the animals. Pr monitory indicators are largely, if not entirely, unknown.

Charles Stockard[1] sought evidence of endocrine influences on the reg lation of growth of dogs or of their different parts. If a conclusion cou be drawn from these studies it would probably be that it is not a qualitati or quantitative difference in the hormonal environment that is inherited regulate the unique somatic differences in dogs but, more commonly, th it is differences in the end organs; thus certain long bones and facial bone presumably conditioned by contrasting hormonal environments, cou coexist in the same body.

Genetic influences may exert their effects upon (1) the endocrine glan producing the hormone that influences the amount, periodicity, or kind hormone produced; (2) those mechanisms that regulate the excretion inactivation of hormones; or (3) those mechanisms that may modify t capacity of end organs to respond to the hormones produced and retaine in the body. These points will be taken up individually.

First, one might ask whether hormonal influences have anything to with cancer and, if so, how the perimeters in which these influences exist a defined. If a carcinogen is defined as a substance whose application resul in the appearance of tumors that otherwise would not have appeared, th some hormones are carcinogenic. Tumors of at least eleven different orga or tissues arise in animals of different species or strains under conditio that implicate hormonal influences in their etiology.[2] If we consider on those tumors that appear in mice of the inbred strains and that are associat with endocrine influences, we must include tumors of the mammary glan uteri, lymphoid tissues, pituitary glands, testes, ovaries, adrenal cortex a medulla, and thyroid. Strain differences exist in the frequency of appe ance of most of these tumors in mice subjected to the influence of hormon

In this paper I shall restrict myself to a discussion of some genetic a hormonal aspects of testicular and pituitary tumorigenesis in mice and strain differences of a quantitative nature in vaginal responses to estroge

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*Pituitary Tumors**

Few untreated mice in our colony have pituitary tumors, even when kept
advanced ages. I have seen but one; it appeared in a mouse with ovarian
anulosa cell tumors.[4] Subsequent to estrogen treatment for prolonged
eriods, pituitary adenomas of nongranular or chromophobic cells appear
equently in mice of one strain, but rarely or never in mice of other strains.[5, 6]
ice of the C57 strain showed a relatively large number of such tumors after
trogen treatment.[5] Subsequently it was found that hybrid mice obtained
y mating mice of the C57 strain with those of the C3H and CBA strains
vhich are not susceptible to pituitary tumors) also acquired pituitary
mors when subjected to estrogen treatment.

These tumors appeared more frequently earlier, and attained larger sizes
males.[6] They did not appear if testosterone propionate, in adequate doses,
as given simultaneously with estrogen. Backcross mice tended to resemble
e predominating heritage.[7] The predisposition to pituitary tumors
ppeared to be conditioned by a dominant gene or genes.

Beyond this point analysis becomes difficult. Prolonged hormonal treat-
ent is required before the tumors appear, and intervening causes of death,
ometimes directly related to hormone treatment, increase the difficulties.
ew tumors appear in mice treated less than 400 days. Furthermore, the
ormone may be acting in 1 of 3 ways: (1) it may be inciting the original
eoplastic change; (2) it may be required for growth of the cells that have
eviated independently of the hormone; or (3) it may act to alter factors
at restrict growth of the tumors and hence indirectly may affect the pheno-
pic manifestation.

The above possibilities have not been subjected to critical test. Some
ads have been obtained, however, by following tumors after cessation of
ormone treatment or after transplantation into other hosts. Every
tuitary tumor we have transplanted that has arisen in an estrogen-treated
ouse has grown only in estrogen-treated hosts, and we have followed
pproximately 50 during the last 17 years, some for as long as 6 years. For
eir growth the tumors depended on estrogen and hence might qualify for
e second possibility mentioned above. However, this conclusion is not
nvincing for 2 reasons: (1) when estrogen treatment is withheld from mice
earing the primary tumors they continue to persist and grow, and (2) after
ansplantation into estrogen-treated hosts the tumors do not grow imme-
ately, but appear only after prolonged latent periods, usually when the
ost begins to show a primary pituitary tumor as well. These observations
n be explained best by assuming that the third possibility mentioned
bove is true, namely, that estrogen may modify the environment in some
ay to permit growth of the tumor.

Some of these possibilities can be attacked experimentally, although this
as not yet been done adequately with the pituitary tumors. All of the

* I shall not refer to all of the studies made on the formation of pituitary tumors or
sticular tumors. An extensive review of this subject has appeared recently in *Cancer*
esearch.[3]

normally occurring estrogens tested, or their esters, and stilbestrol are effec tive in producing pituitary tumors when given in adequate and comparabl physiological doses, but TACE* is much less effective. Pituitary tumor that occur subsequent to thyroid impairment and ionizing irradiation ma not be so strain limited.

In summary, we may say that the tendency of pituitary tumors to appea in estrogen-treated mice is (1) strain limited, and (2) transmitted by bot males and females to hybrid animals, indicating an apparent dominant transmission. At this time we cannot be sure whether the pituitary gland itse is different, the metabolism of the hormone or the action of the hormone o other parts of the body indirectly affecting the pituitary; nor can we b certain whether the hormone incites a primary change in the pituitary, provides growth aids for cells otherwise modified, or modifies the resistance o the body to control pituitary growth. Fewer mice given estrogens of th triphenyl ethylene group have acquired pituitary tumors than those give estratriene estrogens or stilbestrol.

Testicular Tumorigenesis

The strain differences in the appearance of testicular interstitial ce hyperplasias in estrogen-treated mice were first observed in our laborator about 20 years ago.[8] Mice of the A strain given any one of several estrogen failed to show the testicular atrophy such as that observed in mice of othe strains. The testes remained large and became yellowish, and the intertubular spaces filled with interstitial cells. Testicular interstitial cell tumor (ict) were later noted in estrogen-treated mice of this strain.[9-11] Subsequently mice of the Bagg albino strain,[12] from which the Strong A strai originally developed,[13] also showed ict when similarly treated.

Hybrid mice obtained by mating either male or female mice of the A strai with mice of other strains acquired testicular ict after estrogen treatment. In fact, 3 untreated controls of such parentage had ict.[15] The incidenc among the hybrids has never been high, usually between 10 and 35 pe cent.[16] More convincing experiments have not been done because othe types of tumors such as mammary or lymphoid often appeared earlie Nevertheless, the tendency seems to be transmitted genetically as a partia dominant (TABLE 1).

TACE, when injected for long periods of time, has been associated wit the appearance of ict in mice in which these tumors have not appeared aft the injection of other estrogens.[16, 17]

Eight of 27 C57 mice and 46 of 92 hybrid mice (C57 × CBA) develope ict when given TACE for prolonged periods (TABLE 2). None of 15 mi of the A strain similarly treated had ict, although treatment with a relate substance, triphenylethylene, was frequently associated with ict in mice this strain. It is possible that 2 different factors are involved, or that t hormones are metabolized differently in mice of different strains. Exper ments done up to this time have not been extensive enough to exclude t possibility that quantitative differences in estrogen level are of importanc

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* TACE is the synthetic estrogenic substance tri-para-anisyl chloroethylene.

TABLE 1

APPEARANCE OF TESTICULAR INTERSTITIAL CELL TUMORS IN HYBRID MICE GIVEN ESTRADIOL AND ESTRONE, OR THEIR ESTERS, AND STILBESTROL OVER PROLONGED PERIODS[2]

Strain or group	Treatment					
	Estradiol, estrone, or their esters			Stilbestrol		
	No. of mice	No. of tumors	Average age at tumor	No. of mice	No. of tumors	Average age at tumor
A × C57	47	1	350	10	1	468
C57 × A	53	6	495	12	3	411
A × C3H	55	6	486	14	2	434
C3H × A	70	7	413	11	4	399
C57 × CBA	43	—	(431)*	10	—	(561)*
CBA × C57	38	—	(427)*	10	—	(453)*

* The average age of survival of all the mice in the group.

TABLE 2

NFLUENCE OF TRIPHENYLETHYLENE (TPE) AND TRI-*p*-ANISYL CHLOROETHYLENE (TACE) ON THE APPEARANCE OF INTERSTITIAL CELL TUMORS IN MICE OF DIFFERENT INBRED STRAINS OR HYBRID GROUPS[2, 15, 17]

Estrogenic chemical	Strain	No. of animals reported	No. of ict	Duration of treatment (months)	No. of pituitary tumors
TPE	JK*	13	7	8–16	
TPE	A	17	7	15–18	
TPE	C3H*	6	1	9–22	
TPE	C12I*	5	0	18–25	
TPE	N*	5	0	13–21	
TACE	CBA*	17	0	12–22	0
TACE	A	15	0	10–15	0
TACE	C57*	27	8	11–19	0
TACE	C3H*	14	0	12–18	0
TACE	BC*	37	0	10–21	5
TACE	BC × C57*	27	0	10–27	17
TACE	A × C57	14	10	15–23	0
TACE	A × C3H	24	4	11–32	0
TACE	C57 × CBA*	92	46	8–28	5

* Mice of these strains have not had ict after injections of stilbestrol or estradiol benzoate. Mice of the strains or groups not so designated have ict when given stilbestrol or estradiol benzoate.[2]

Mice of the BALB/C strain seem to be especially susceptible to the formation of ict after estrogen treatment.[18] Mice of one strain[19] frequently hav ict without the injection of exogenous hormones, but the appearance o mammary cancer in males of this strain indicates an unusual intrinsi source of estrogen.

At one time it was thought that the testicular tumors arose becaus estrogens modified pituitary function; the assumption was made that th pituitary glands of mice of different strains were affected differently.[8, 1 This assumption has never been proved. Ict and pituitary chromophob adenomas have been coexistant on a few occasions, indicating that th 2 responses are not mutually exclusive.[14]

The differences in response do reside in the testes, however, as has bee demonstrated recently.[20] When testes of mice of the A strain were transplanted subcutaneously into hybrid mice (A × C3H) that were subsequently treated with estrogens, they acquired ict. Testes of mice of the C3H strai similarly transplanted did not become tumorous (TABLE 3). Such teste were presumably in a comparable hormone environment. This does no mean that pituitary hormones are not involved, but it certainly means tha strain differences in the effects of estrogens on the pituitary gland need no be assumed.

TABLE 3

TUMORS IN TRANSPLANTED TESTES IN MALE MICE GIVEN STILBESTROL-CHOLESTERO

(1 PART TO 3 PARTS)

Testes of Newborn Mice Were Transplanted in Hosts 29 to 108 Days of Age[20]

Donor	Host	Number	Number of tumors
A	A	9	3
C3H	C3H	12	1 Nodule
A	(A × C3H)	42	26
C3H	(A × C3H)	49	1
(A × C3H)	(A × C3H)	14	4

Again we have no critical evidence as to whether estrogen acts (1) a a carcinogen in inciting directly or indirectly the neoplastic change in th testis or (2) as a growth requirement for deviated cells. Certainly most o the ict are dependent upon estrogen for their growth when they first appea Some ict regress if treatment is withdrawn after they have appeared,[18] bu may subsequently reappear and grow progressively. After transplantatio most of the ict in mice of the Strong A strain grow only in estrogen-treate hosts. They may remain dormant for months in the absence of estrogen an then promptly begin to grow when estrogen is added.[21] After prolonge transplantation most tumors begin to grow in the absence of estrogen.

The changes from normal to abnormal to autonomous seem stepwise, eac step requiring time that can be measured in months or even years.[11] If th changes are temporally progressive in a continuous sequence, then we a recognizing only separate stages in the continuation—hence their stepwise appearance. Some of the neoplasms may be in part ontogenically manifest. They may be determined by the genotype, the hormones only modifying the age of phenotypic manifestation.

Vaginal Sensitivity to Estrogens

Differences exist in the sensitivity of end organs to hormones as determined by non-neoplastic responses as well. Loeb and his associates[22] noted differences in the frequency and duration of vaginal cornification in mice of several strains. Such differences could be due to differences in pituitary or ovarian function, or both. Subsequently several investigators noted strain differences in the vaginal response of ovariectomized mice of different strains to injected estrogens.[23, 24] These differences might be due to (1) strain differences in the sensitivity of vaginal epithelium, (2) strain differences in the mechanisms of destroying or excreting estrogens, or (3) strain differences in the presence or absence of inhibiting or augmenting substances. Mühlbock[23] rather convincingly excluded the second possibility by showing that the topical application of estrogen disclosed comparable strain differences. This approach would not separate the first and third possibilities.

Hybrid mice derived from strains with contrasting vaginal sensitivities to estrogen show intermediate and usually more consistent sensitivities. The vaginas of mice of pure strains will transplant well in their hybrid offspring. Hence it is possible to get a vagina from a strain sensitive to estrogen and one from a strain of low sensitivity in the same environment.[25] Vaginas of mice of the A and the C57 strains have been transplanted into hybrid hosts (AC). Also, vaginas of A donors have been put in A hosts, C57's in C57 hosts, and AC's in AC hosts. The transplanted vaginas do not respond to injected estrogens as well as do intact vaginas; about twice as much estrogen is required. The vaginas of C57 donors are twice as sensitive to estrogen as those of A donors, even in the same host. This offers evidence that strain differences lie in the vaginal tissue itself and not in the presence of augmenting or inhibiting substances in the body. It does not, however, prove the first point, because ontogenic experiences prior to transplantation may condition the tissues by inducing irreversible changes. For example, it is known that androgens given prenatally will prevent the development of the lower vagina. We must assume the possibility that end organs may be modified with respect to responses to hormones by earlier ontogenic experiences, the latter in turn being genetically determined.

Summary

Some of the evidence that genetic factors determine qualitative differences in responses to hormones that result in or lead to neoplasia has been presented, with special reference to the pituitary gland and testis. The susceptibility to tumors of the pituitary gland and testis was rarely apparent in untreated mice, and appeared after prolonged periods of estrogen treatment in mice of some strains, but not of others. Predisposition for these tumors is transmitted by both male and female parents to their hybrid offspring.

The tumors are usually hormone dependent when transplanted to related hosts but, after becoming established, the testicular tumors are hormone independent. It is possible that the tumors may produce, when they attain suitable sizes, products that will contribute to their growth.

Rather convincing evidence shows that the genetic predetermination for testicular tumors in mice of the A strain is in the testis and not in systemic differences in hormone levels or in the production of pituitary hormones. That more than one mechanism may be involved in testicular tumorigenesis is indicated by strain differences in responses to different estrogens. The possibility of quantitative differences in susceptibility has not been excluded completely, however.

Quantitative differences in responsiveness of the vaginal mucosa to estrogens have been demonstrated to be in the vaginal epithelium, and are not due to differences in the metabolism of estrogen or the presence of augmentors or inhibitors.

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RELATIONSHIPS, ESTABLISHED AND PROSPECTIVE, BETWEEN VIRUSES AND CANCER

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The first direct causal relationship between cancer and a virus was established experimentally in 1908 by Ellermann and Bang in their work on the transmission of fowl leukemia by means of cell-free preparations. Unfortunately, this important experiment failed to have the impact on contemporary investigations that it should have had because the neoplastic nature of this disease was not then clearly recognized. However, shortly thereafter, in 1911, Peyton Rous, working at the Rockefeller Institute for Medical Research, New York, N. Y., found that a sarcoma that appeared spontaneously in a Plymouth Rock chicken could be passed from chicken to chicken by means of cell-free filtrates. Clearly, here was a malignant growth caused by a virus or viruslike agent. At first, chickens closely related to the chicken in which the sarcoma originated were necessary for successful transmission, but later this virus was adapted to other kinds of chickens and to other types of fowl such as ducks, turkeys, and pheasants. In some cases this involved several passages in the new host by means of cells before it became possible to pass the sarcoma by means of cell-free filtrates. Although for some years this caused much doubt regarding the true relationship between the virus and the sarcoma, the Rous virus did serve to set the pattern, and today tumor viruses of chickens and other avian species are accepted without question. In addition, the situation with respect to fowl leukemia has been clarified somewhat, although areas of uncertainty remain. Beard (1957) and his associates have concentrated and purified two distinct viruses, one responsible for myeloblastic leukemia and the other for erythroblastic leukemia. Burmester (1957) has obtained evidence indicating that lymphomatosis is characterized by a group of related viruses. It is possible that all or most of the avian leukemia and tumor viruses, including the Rous virus, may be related more or less closely.

In 1932 Shope found a fibroma of rabbits that was caused by a virus. Later, Berry and Dedrick (1936) reported that inoculation of a mixture of heat-inactivated myxoma virus and active fibroma virus resulted in myxomatosis. This very interesting situation is still greatly in need of further experimentation. In 1940 Duran-Reynals noted that inoculation of newborn rabbits with small doses of fibroma virus caused tumors or generalized fibromas, whereas larger doses of the same virus caused necrotic inflammatory lesions not very different from those caused by myxoma virus. This work was the forerunner of much important later work of a similar nature with still other viruses by Duran-Reynals.

In 1933 Shope discovered in our native wild cottontail rabbit the now famous papilloma virus of rabbits. This virus causes a benign tumor or papilloma in the wild rabbit that only occasionally develops into a malignant growth with metastases. However, inoculation of domestic rabbits is followed by the development of papillomas that, unlike those in wild rabbits, appear to contain no infectious virus and almost always progress into cancers in which there still seems to be no infectious virus. Immunological evidence for the existence of virus in the domestic rabbit papillomas and cancers has been obtained by Smith *et al.* (1952). Furthermore, Rous and Friedewald (1944) have found that the cancers derived from papilloma virus-infected rabbit cells on treatment with chemical carcinogens or tar were of the same type that arise from these cells spontaneously after a much longer time. This work would seem to indicate that the chemical carcinogens acted by way of the virus. The rabbit papilloma virus has been concentrated and purified, and many of its biochemical and biophysical properties have been studied. Needless to say, the unusual opportunity provided by this system to study the transition from a benign virus-induced papilloma to a malignant growth has been and is still being extensively investigated. During recent years papillomas of the skin and oral mucosa of several different animals, such as horses, cows, cats, dogs, and rats, have been found to be transmissible by means of filtrates. Unfortunately, these have been little studied; hence the extent as well as the importance of these viruses remains in doubt. A very interesting virus discovered in 1955 by Shope causes fibromas in deer.

In 1934 Lucké reported the isolation of a filtrable agent from an adenocarcinoma of the leopard frog. It is of some interest that inoculation of this virus into distant sites of the leopard frog yielded no local tumors, but did yield kidney carcinomas, and that inoculation of other kinds of frogs yielded no tumors. In 1936 Bittner discovered in the milk of mice the agent that seems to be responsible for the development of breast cancer in mice many months after inoculation of the agent. However, genetic and hormonal factors have been found to play an exceedingly important role, so much so that some situations are known in which breast cancer is present without demonstrable virus and others in which virus can be inoculated without the subsequent appearance of breast cancer. While there is no doubt with respect to the existence of a virus that can cause breast cancer in mice under specified conditions, our knowledge of the interplay between viral, genetic, and hormonal factors leaves much to be desired.

Black, in 1945, described a leafhopper-transmitted virus that would cause tumors on the roots of sweet clover plants, as well as in many other plants. The tumors caused by this wound-tumor virus were found to differ from the outgrowths known earlier in certain tobacco and sugar cane diseases in that they seemed capable of indefinite growth as unorganized tissue. Black has pointed out that striking similarities exist between the wound-tumor disease and mammary cancer in mice, in that a virus plays an important role in each; genetic and hormonal factors, however, can have a tremendous influence on the virus-host reaction.

In 1951 Gross reported that the inoculation of newborn mice of strains free of leukemia with cell-free filtrates of extracts of leukemic organs from mice of a high leukemic strain resulted in leukemia within one or two years. Several investigators have devoted much time and effort to studies of this mouse leukemia, and it now appears likely that several viruses causing different kinds of tumors are actually involved and that the genetic factors and age of the host play a much more important role than was suspected earlier. The separation and recognition of the different viruses or virus strains and their corresponding diseases and the elucidation of the role played by genetic factors represent problems of the first magnitude. However, today there seems to be no doubt that these mouse viruses can cause cancer and this is, of course, the important fact for the present discussion.

Friend (1957) has reported the discovery of a filtrable agent that will induce leukemia in mice of any age within as short a period as two or three weeks after inoculation. This remarkable agent was found during the course of studies on the effect of cell-free preparations of the Ehrlich ascites tumor in infant Swiss mice. Since infant mice are not necessary for experiments with this agent, and since the disease develops rapidly, this virus would appear to possess definite advantages for exploratory experimentation over the viruses that Gross has investigated.

I trust that this very brief review of cases in which there is good experimental proof for a direct causal relationship between viruses and cancer in animals and plants will be sufficient to ensure acceptance of the fact that viruses can cause cancer. I think it is necessary, next, to realize that basic biological phenomena generally do not differ strikingly as one goes from one species to another and therefore that the fact, now proved beyond contention, that viruses can cause cancer in animals is directly pertinent to the human cancer problem. Recent advances in the cultivation of human cells *in vitro* and especially the newer knowledge of certain properties of viruses warrant a marked change in our present thinking on the problem of human cancer. I believe the time has come for us to assume that viruses are responsible for most kinds of cancer and to design and execute our experiments accordingly. Cancer is basically a problem in growth, and there is no reason to believe that the growth of most human cells is fundamentally different from the growth of most animal cells. Acceptance of the viral etiology of human cancer as a working hypothesis will involve a marked change in attitude on the part of many investigators, but this is necessary if the right approach and the right design of experimentation are to result. What we do depends in large measure upon what we think. Now let us examine some of the prospective relationships that may influence our thinking.

Since there is no evidence that human cancer is infectious, many investigators believe that, because viruses are infectious agents, they cannot possibly be of etiological importance in human cancer. However, this is not a valid conclusion because it is well known that animal tumor viruses may alternately be filtrable and infectious and then nonfiltrable and noninfectious due apparently to variations in the amount of virus present. It is also well known that viruses may be highly specific; so specific, in fact, that a given virus will infect and cause disease only in one kind of cell in one kind of animal, and therefore under many conditions may appear noninfectious. Inoculation of the mucous membrane of a susceptible rabbit with the Shope rabbit papilloma virus yields no tumors. Under these conditions this virus would appear to be noninfectious. Even the Rous sarcoma virus when first solated would cause cancer only in blood relatives of the chicken from which was obtained. Later on, this virus was adapted to other chickens and o other types of fowl, such as ducks and pheasants. In some cases this nvolved several passages in the new host by means of cells before it became ossible to pass the tumor by means of cell-free filtrates. In order to transnit a virus from a given host to a more distantly related host it is usually ecessary to adapt first to an intermediate host. There would be, for xample, little expectation that a virus long adapted to existence in human ells would grow readily in most animal cells. However, the fact that some uman viruses have been found to grow in certain animal cells indicates that he situation is not completely hopeless and, therefore, that the search should ontinue. Nevertheless, it should be obvious that great difficulties might be nvolved in demonstrating the existence of a virus that is actually present n a human cancer. Certainly, the techniques that have proved useful in emonstrating the existence of certain animal cancer viruses have not yet een used in experiments with human cancer. Until these techniques have een used exhaustively, without success, it would be foolish to disregard the ole that viruses may be playing in human cancer.

Needless to say, the cultivation of human cells *in vitro* opens up wonderful xperimental possibilities. The work of Earle (1943) on tissue cultures and f Gey and his associates (1952) on HeLa cells has stimulated great activity n the cultivation of human cells. In our own laboratory we have about a ozen different kinds of normal and malignant cells of human origin under ultivation. While most of these are being used for studies on the effects f known viruses, it is obvious that such cells can be examined for the presence f hitherto unknown, as well as known, viruses or can be used as test mateials for suspected human viruses. The human amnion cell, which my olleagues Zitcer, Fogh, and Dunnebacke (1955) first obtained from the fullerm amnion in cell culture, is proving of great use in this connection, as ell as in studies on the transition from a normal to a potentially malignant ell. For example, we are finding interesting changes in chromosome number nd in the ability of these human amnion cells to grow in cortisone- or X-rayreated animals as they are passed in culture. Many other laboratories ave normal and malignant human cells under cultivation, and significant ew findings may be expected to result from this new and, potentially, xtremely powerful experimental approach.

Another fact of importance, first recognized with the Rous sarcoma virus, that a given tumor may be transmissible, for a time, only by the inocution of living cells, and later by means of cell-free filtrates. The fact that his occurred with the Rous sarcoma was used as an argument against the iral causation of this tumor for a long time. However, similar results were btained in the case of other tumors, and it was thought that the variation transmissibility was due to differences in the actual amount of virus prouced. Recently Carr (1943) and Duran-Reynals (1957) proved experientally that the amount of virus present in the tumor tissue is dependent pon the age of the host and the age of the tumor; in general, the younger the ost and the younger the tumor, the more virus can be extracted. However, it should be noted that Duran-Reynals found that inoculation of Rous virus to newly hatched chicks results in a hemorrhagic, rather than a neoplastic response. Bryan and his co-workers (1955) made a thorough study of the relationship between the amount of Rous virus inoculated and the amoun of virus extractable from the tumors at different times. It was found tha the extractable virus approached a maximum when relatively strong inocula of about two hundred and fifty 50 per cent-effective doses, were used, bu that at lower dosage levels the amount was highly correlated with the dose They also found that very small doses of virus yielded tumors from whic no virus was recoverable by extraction.

Beard (1956) has assembled evidence to show that differences in the infec tivity of Shope virus papillomas of wild cottontail and of domestic rabbits generally ascribed to latency or masking in the latter, are due, not to differ ences in the nature of the virus, but to wide differences in the actual amoun of virus present in the papillomas. The rate of virus production within cell may vary so much that, in some cases, hundreds to hundreds of thousand of virus particles are produced per cell whereas, in other cases, the rate o virus production may approach, or may be the same as, the rate of cell divi sion. In the former case the diseased tissue or tumor would be expected t yield large quantities of virus and hence represent a highly infectious situatio whereas, in the latter case, the diseased tissue or tumor might be regarde as noninfectious because of the lack of demonstrable virus. Changes i nutrient or in temperature during the period of virus growth can cause drasti changes in the amount of a virus present in a host. It is obvious, therefore that before one should conclude that a given tumor is nonviral in nature, one should be certain that the extract has been tested on cells known to be susceptible and to yield a demonstrable reaction with the virus; also that the virus is absent in fact, and not just present in provocative yet subinfectiou amounts. Needless to say, it is well known that proper concentration procedures may convert subinfectious preparations into infectious preparations

One sometimes hears the argument that cancer viruses do not exist i human beings because, if they did, someone would have found them by thi time. Attention might have been given to this argument some years ago but not now, for literally dozens of hitherto unknown human viruses hav been discovered during the past year or so. The typing program for poli viruses, during which extracts of stools from hundreds of patients were use to inoculate monkey kidney and occasionally other cells in tissue culture resulted in the discovery of dozens of enteric viruses known as the ECH viruses. Another group, consisting of many viruses isolated in a simila fashion, but from the upper respiratory tract of man, are the adeno-viruse (formerly known as the APC or ARD viruses). A third group of viruse has been isolated from samples of human sera. Thus we have today mor than a sufficiency of human viruses; accordingly, now there is certainly n reason why viruses should not be considered as causative agents in cancer f lack of the viruses. Actually, these recent developments lead one to suspe that many more undiscovered viruses are present in presumably norm human beings.

The finding that adenoviruses had a cytopathogenic effect on a human cancer cell, the HeLa cell, suggested to Huebner and other scientists (Suskind *et al.*, 1957) at the National Institutes of Health in Bethesda, Md., that these viruses might have some effect on similar cells in human beings. It was found that the injection of these viruses into a cervical cancer mass or into the blood stream resulted in the disintegration and disappearance of most of the cancerous mass. However, all of the cancer cells were not destroyed by the time neutralizing antibodies to the virus developed and these served to protect the residual cancer. Earlier work by Southam and Moore (1952) had shown that the inoculation of human beings carrying a cancer with certain neurotropic viruses sometimes caused regression of the cancer. Work of this general nature has only begun, but the fact that infection of certain cancer cells with certain viruses causes the destruction of the cancer cells is most significant. Because of the well-known plasticity of viruses it should be possible, by means of already known passage techniques, to develop viruses having no effect on normal cells, but with a special predilection for the destruction of cancer cells. Several laboratories are already engaged in work of this type.

It has long been known that cancer could result from prolonged exposure to coal tar and, since 1920, extensive studies on chemical carcinogens have been made. These have been divided into locally acting and remotely acting carcinogens, the former causing cancer at the site of application and the latter causing tumors in specific organs or tissues, regardless of the site or manner of application. It has generally been assumed that the carcinogen has caused some change in a cell that then progresses to cancer, but a few investigators have suggested that the chemical carcinogen works by activating or by causing a mutation in a latent virus. Rous and Friedewald (1944) have made extensive studies on the effect of chemical carcinogens on virus-induced rabbit papillomas, and their evidence would seem to indicate that the chemical carcinogens acted by way of the virus. Duran-Reynals (1952) found that, in almost every case, treatment of chicks, adult chickens, and pigeons with methylcholanthrene activated a latent or subinfectious fowl pox virus that produced lesions, and these subsequently progressed to cancer. His work certainly focuses attention on the question of whether an ordinary virus such as fowl pox can be a potential cancer virus.

In view of the great variety of carcinogens, which range from hundreds of different kinds of chemicals to physical agents such as ultraviolet or X irradiation and mechanical effects such as continued abrasions or irritations, the supposition that cancer results from some change within the cell is quite reasonable. However, if cells have viruses as passengers within them and if viruses can cause cancer, it is just as reasonable to assume that these diverse agents are affecting the virus as it is to assume that they are affecting the genetic apparatus of the cell.

The discovery of many hitherto unknown viruses in human beings and the possibility that such viruses may persist within the cells of man for years, if not for a lifetime, is really not too surprising. It is well known, for example, that most human beings acquire the virus of herpes simplex quite early in life and, in many persons, evidence for the persistence of this virus throughout their lifetimes is strong. Traub (1939) has found that infection of a mouse colony with the virus of lymphocytic choriomeningitis can result, with time, in an inapparent infection of all animals. The virus is apparently transmitted *in utero* and remains with the animal throughout its life; this virus accordingly persists through generation after generation of mice. It is interesting to note that injection of mice from such a colony with sterile broth can revive the pathogenicity of the virus. Meyer (1942) has shown that psittacosis virus can persist in the form of an inapparent infection in groups of parrots and certain other birds for long periods of time, only to have frank disease appear following unusually crowded conditions. Shope has shown experimentally that swine influenza virus can remain in a masked or latent form in the intermediate host, the swine lung worm, for very long periods of time. Certain potato viruses, such as potato X virus, also known as the healthy potato virus or the latent mosaic of potato virus, can be passed from generation to generation without causing apparent disease. This virus is not present in several varieties of potato grown in Europe, but it is thought to be present in all, or almost all, potato plants grown in the United States. Needless to say, it was only by virtue of the fact that potato plants without this virus are known to exist and the fact that this virus causes obvious disease symptoms when inoculated to certain other plants that it was possible to establish its actual existence. In the absence of this information this latent mosaic virus would have to be regarded as a normal constituent of the potato plant. Several examples of the persistence of a plant virus in its insect vector are known. Clover clubleaf virus has been passed by Black (1950) for 21 generations of its insect vector during a course of 5 years; rice stunt virus for 6 generations by Fukushi (1940); and aster yellows virus for 10 passages in a leafhopper by Maramorosch (1952). The persistence of a virus in man for many years, for a lifetime, or even for 2 or more generations, should not be regarded as an unexpected or unusual phenomenon, but rather in complete accord with a well-established biological principle.

The fact that viruses may persist in cells for generations provides ample reason for us to consider the relationships between viruses and genes since, under these conditions, both genes and viruses are passed from generation to generation of cells, and there is something in the cancer cell that also is passed from cell to cell. Muller's (1947) estimate of the maximum size of a gene would place it, with respect to size, just below tobacco mosaic virus, near the middle of the viruses. Both genes and viruses seem to be nucleoproteins, and both reproduce only within specific living cells. Both possess the ability to mutate. Although viruses generally reproduce many times within a given cell, we know of some situations in which they appear to reproduce only once with each cell division. Genes usually reproduce once with each cell division, but here also the rate can be changed, as, for example, in the case of polyploidy resulting from treatment with colchicine. Actually, the similarities between genes and viruses are so remarkable that viruses very early were referred to as "naked genes" or "genes on the loose." Two discoveries, one made in 1928 (Griffith, 1928) and the other in 1952 (Zinder and Lederberg, 1952), have provided experimental evidence for an exceedingly intimate relationship between viruses and genes. Griffith found that he could transform one specific S type of pneumococcus into another specific S type by injecting mice with nonvirulent R forms together with large amounts of heat-killed S pneumococci of a type other than that of the organisms from which the R cells were derived. Living virulent S organisms of the same type as the heat-killed S forms were then recovered from the animals. Later Dawson and Sia (1931), as well as Alloway (1932, 1933), found that the addition of an extract of one type of capsulated pneumococcus to a culture of a noncapsulated rough form would convert the latter into the same type of capsulated pneumococcus that provided the extract. It was obvious that something was being transferred and, in 1938 (Stanley, 1938), I suggested the possibility that this something might be a virus. In 1944 Avery and his colleagues at the Rockefeller Institute proved that this something was a transforming principle consisting of desoxyribonucleic acid (DNA). Muller, in 1947, discussed the possibility that the DNA might correspond to still viable parts of bacterial chromosomes loose in solution that, after entering the capsuleless bacteria, undergo a kind of crossing over with the chromosomes of the host, but this suggestion was not widely accepted. That the phenomenon was not an isolated one was demonstrated in 1953 by Leidy *et al.*, who obtained similar results with an influenza bacteria system. The close relationship to genetics was further emphasized by the work of Hotchkiss (1955) and of Ephrussi-Taylor (1955), as well as of Leidy *et al.*, who showed that drug resistance and other genetic factors could be so transferred. This work provided evidence that genetic factors or genes, if one prefers such a designation, can be represented by DNA and can be obtained in chemically pure solution.

This information, and also our knowledge of viruses, was soon fortified by the very important discovery of Zinder and Lederberg in 1952 of transduction in *Salmonella* by means of a bacterial virus. It was found that genetic factors could be carried from one type of *Salmonella* cells to another by means of a bacterial virus. In this type of transformation the genetic fragment is not free, but is carried within the structure of the bacterial virus. It is not affected, for example, by the enzyme desoxyribonuclease and, in this respect, it is unlike the DNA pneumococcus-transforming principle. However, it is not necessary for the virus actually to possess virus activity, for killing of the virus by ultraviolet light does not prevent the transduction of other traits. The closeness of the relationship between the virus and the genes of the host is emphasized by the fact that the transducing ability of any bacterial virus is determined strictly by the character of the cells on which the virus was most recently grown. Virus grown on serotype E_2 *Salmonella* cells, when added to serotype E_1 cells, will convert a fraction of these cells into serotype E_2 cells. It is of interest to note that the virus in filtrates of toxin-forming bacterial strains will convert nontoxin-forming cells into toxin-forming cells. In transduction, a fragment of a chromosome that might be regarded as a gene, or as a collection of a few or even many genes, can be transferred from one kind of donor cell to another kind of receiver cell and be incorporated into the genetic apparatus of the receiver cell. In the pneumococcus or influenza bacterium this can be caused by a DNA preparation that can be separated and isolated as such; in *Salmonella*, this gene or gene collection rides within the bacterial virus, presumably with the viral DNA, which is added to the cell to be transduced. Here one hardly knows what to call a virus and what to call a gene, for it is obvious that at times the two merge completely.

For the purposes of the present discussion I direct your attention to the persistence of a bacterial virus in the form of a prophage in lysogenic strains of bacteria, and suggest that this might provide a most significant experimental model for the process I have in mind. Lysogenic bacteria perpetuate, in what may be considered a hereditary manner, the property of being able to produce a bacterial virus, a phenomenon that has been investigated extensively by Lwoff and his associates (1950). The term prophage is used to describe the form in which the potentiality to produce a bacterial virus is perpetuated in lysogenic bacteria. Prophage is nonpathogenic and noninfectious in the usual sense but, since it is multiplied at least once with each cell division, it may be regarded as infectious in the sense that genes or chromosomes are infectious. In other words, the prophage might be considered as a temporary part of the genetic apparatus of the cell, the genetic element that differentiates a lysogenic from a sensitive cell and, at the same time, as the noninfectious form of a bacterial virus. When prophage develops into the bacterial virus the bacterium is destroyed; therefore, a lysogenic bacterium survives only if it does not produce the bacterial viruses.

Of greatest importance is the fact that treatment of certain lysogenic strains of bacteria with physical and chemical agents, such as X rays, ultraviolet light, nitrogen mustard, certain reducing agents, or iron chelating agents, results, after a latent period, in the lysis of the bacterial cells and the release of large amounts of bacterial virus particles. These agents are called "inducers," and some are recognized as carcinogenic agents for man and animals. Nonlysogenic bacteria are, of course, well known, and these are unaffected by these "inducers" insofar as the production of a bacterial virus is concerned. It may be noted also that there is at least one well-established case in which a strain of lysogenic bacteria, *B. megatherium*, has been converted into a nonlysogenic strain. This fact can be regarded as evidence for the belief that the prophage and the resulting bacterial virus are something other than normal components of the cell. It may also be concluded that the evidence indicates that there are times when a specific genetic element of a cell can be freed of the normal controlling mechanism of the cell and go forth in viable form in solution or associated with a virus, enter a different cell, replace a homologous chromosomal segment, and resume its original specific function in the new cell. It is obvious that the latter phenomenon could readily be considered an infectious process and that, under certain circumstances, viruses can act as genes and genes as viruses.

I now mention a recent very important discovery made in our laboratory by Fraenkel-Conrat (1956), since this finding may prove quite pertinent to a consideration of prospective relationships between viruses and cancer. It was reported by Fraenkel-Conrat (1956), and also shortly thereafter by Gierer and Schramm (1956) in Germany, that special treatment of tobacco mosaic virus yielded a nucleic acid preparation possessing virus activity. It would now appear necessary to recognize that a nucleic acid structure, perhaps as small as 300,000 in molecular weight, can possess, coded within its 1000 or so nucleotides, not only all of the information that is necessary to bring about in the host cell the production of more of this same nucleic acid but also, apparently, the *de novo* synthesis of its own characteristic and highly specific protein with which it eventually coats itself. This work provides wonderful evidence for a direct relationship between specific nucleic acid and specific protein synthesis, and it makes it possible to consider virus and gene action, including their relationships to cancer in terms, not of nucleoprotein structure, but of nucleic acid structure.

It may be calculated that a 1000-unit polynucleotide linear chain consisting of a coded repeat of only 4 different components, adenine, guanine, cytosine, and uracil, in the same ratio that exists in tobacco mosaic virus nucleic acid, could form about 10^{590} different arrangements. This number is so large as to be incomprehensible. Even a 100-unit polynucleotide chain of this composition could exist in about 10^{57} different arrangements; a number vastly larger than the total of all living things on earth and in the oceans. We have, therefore, in this structure consisting of the 4 chemicals, adenine, guanine, cytosine, and uracil (thymine in the case of desoxyribonucleic acid), repeated many times over in unique fashion, the code for every bit of life on earth and in the sea. When a normal cell becomes a cancer cell there is undoubtedly a change in this structure within the cell. It is of interest to note that many anticancer compounds are antimetabolites for these chemical components of nucleic acids. Furthermore, in our laboratory Litman and Pardee (1956) made the very important observation that the incorporation of 5-bromouracil into a bacterial virus in place of thymine resulted in the production of the highest percentage of mutants ever recorded.

It would now appear wise to revise the generally accepted definition of a virus to include nucleic acids; perhaps also to include replicating structures that do not evidence infectivity in the usual sense because, normally, they are duplicated only once or a minimal number of times during each cell division, and may never leave the cell during many generations. Such a viral nucleic acid might temporarily appear to be a part of, or associated with, the genetic apparatus of the cell, but remain subject to chemical or physical stimulation or shock that could cause it to mature, increase greatly its rate of replication, perhaps mutate but, in any case to separate and act as an independent functional unit. Because of the long history of viruses indicating that they are exogenous in nature, and in order to differentiate such a replicating structure from the normal components of the cell, it is imperative, if we are to preserve present concepts, that initially the structure or virus must have entered the cell from without. I hope that, in order to avoid utter confusion in our concepts, the experimental results will continue to be compatible with the idea that viruses are exogenous in nature, even though, as already indicated, we occasionally come very close to the borderline between an exogenous and an endogenous origin. Thus, while the reproduction of genetic units from a member of one species in a member of another species might be accepted (certainly the transduction phenomenon makes such transfer appear possible and even logical), the aberration of a normal component of a given host to yield something that would then reproduce in that host would be incompatible. So far, however, it has not been necessary to trespass for, in every case examined carefully, the evidence that the virus or self-duplicating mechanism has entered the cell from without at some time in the immediate or distant past is good.

Now, of course, there are direct approaches to the human cancer problem that should be continued and even intensified in view of several interesting experimental results. Two groups of investigators, Dmochowski and Passey (1952) and Gross and his co-workers (1952), using the electron microscope, have concluded that more particles sedimentable by means of high-speed centrifugation are present in breast tumors and milks from women with breast cancer than in the milks from apparently healthy women. Bostick (1957) has evidence for the presence of a filtrable agent capable of causing disease in mice in extracts of lymph nodes from persons suffering from Hodgkin's disease. Schwartz *et al.* (1957) have also reported experimental evidence for a filtrable agent in the brains of persons dying of leukemia that will cause leukemia in mice at a time when, normally, there would be no leukemia. Dmochowski and Grey (1957), as well as others, have obtained electron micrographs of thin sections of lymph nodes of children suffering from leukemia that show particles similar to those found in similar sections from leukemic mice. Whether or not these particles, as well as others seen in electron micrographs of thin sections of centrifugally concentrated extracts of different kinds of human cancer tissue, actually represent viruses is, of course, not known. Unfortunately, there is a current tendency to read too much into such findings. It must always be remembered that the cardinal attribute of a virus is its ability to cause replication and, in the absence of proof that a given characteristic particle can bring about replication of itself, the particle should not be referred to as virus. The approach involving the direct examination of human material will therefore encounter many difficulties but, in view of the great advances in tissue or cell culture techniques, I believe not only that the approach should be made but that the activity in this area, especially in leukemia of children, should be intensified. However, there is a strong possibility that human cancer, in part, may require new and different approaches, and it is for this reason that attention should be devoted to the prospective relationships that are discussed in this paper. The relationships involved in animal, bacterial, and plant viruses should be examined carefully for biologically analogous situations that may obtain in human cancer. Certainly we know that man has coursing through his body many viruses that were unknown a few years ago, and we do not know what many of them are doing there. We know that viruses can persist in their host for generations, in either an infectious or a noninfectious form. We know that viruses can mutate to form new strains that cause different disease symptoms. We know that viruses may have different effects depending on the age, genetics, and state of nutrition and hormonal balance of the host. We know that different carcinogenic agents can activate subinfectious or latent viruses or prophages with consequent cellular destruction. It would certainly appear that the activation of prophages by certain chemical or physical agents, with development into a fully infectious bacterial virus and the consequent destruction of the bacterial cells, as well as the phenomenon of transduction by free desoxyribonucleic acid in the pneumococcus and by bacterial viruses in *Salmonella*, is pertinent to the human cancer problem, especially in view of the recent discovery of dozens upon dozens of new viruses of man. It is difficult to escape the conclusion that viruses may be the etiological agents for most, if not all, cancer, including cancer in man, and that this represents by far the most intellectually satisfying working hypothesis consistent with all presently known facts. Of the greatest importance is the fact that acceptance of viruses as etiological agents in cancer generally as a working hypothesis will stimulate new experimental approaches to the cancer problem, which have been long neglected and which are only now beginning in several laboratories. Could it be that the recent discovery of dozens of hitherto unknown viruses of man that seem to be coursing through the human body from time to time is but the forerunner of the discovery that specific nucleic acids within our cells, perhaps even of exogenous genetic origin and acquired by transduction or a related phenomenon, have a relationship to our cancers? Needless to say, what we do in the way of experimentation depends in large measure upon what we think, and I am sure the time has come when we should change our outlook with respect to the nature of cancer.

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Discussion of the Paper

ALBERT LEVAN (*University of Lund, Lund, Sweden*): The viral and mutative nduction of malignancy are not alternative but complementary hypotheses. The mechanism of cancerogenesis studied in tissue cultures involves the complete breakdown of the normal genotype and the formation of new genotypes with malignant properties. We do not need to assume any virus action in connection with this method of induction, although, of course, a virus nfluence cannot be disproved.

Little is known about the chromosome conditions of cancerogenesis by virus action, but certain facts indicate that the genotypic changes are different in type.

EVIDENCE FROM EXPERIMENTALLY INDUCED NEOPLASMS RELATIVE TO THE MUTATION HYPOTHESIS OF THE ORIGIN OF MALIGNANT DISEASE

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The experimental production of sarcoma of the liver of rats was reported in 1920 by F. D. Bullock and M. R. Curtis. Discrete localized areas o malignant disease were observed in the walls of cysts surrounding the parasites *Cysticercus fasciolaris* 8 to 27 months after the rats had been fed egg of *Taenia taeniaeformis*, the common tapeworm of the cat. In rats experimentally infected with one to one hundred parasites, usually only one cys and, in early tumor bearers, only a small part of the cyst wall was primarily involved in the malignant transformation. Subsequent analyses[2] of data on nearly 4000 tumor-bearing rats revealed (TABLE 1) that the average laten period preceding onset of the malignant complication decreased from 18.8 ± 0.12 months for rats with a single parasite to 12.2 ± 0.31 months fo rats with 60 parasites and the percentage of rats with induced tumors increased (TABLE 2), respectively, from 30 to nearly 100.

From these observations it appeared that an increase in the surface area of tissue exposed to the incitant decreased directly the average time interva necessary for the onset of the malignant change and increased directly the probability of its occurrence. Further, the occurrence of neoplastic disease in the walls of *Cysticercus* cysts was found[3] to be distributed fortuitously to the several lobes of the liver as shown in TABLE 3. The morphology of the observed neoplasms corresponded to the types of cells found in the cyst wall, each being represented by a number consistent with the supposition that the change from a normal to a cancer cell resulted from the chance action of the incitant.

Previously demonstrated genetic differences[4] in the occurrence of *Cysticercus*-induced sarcomas were defined[2] as differences in the host's susceptibility to *Cysticercus* infestation and in longevity or resistance to common laboratory diseases. Strains of rats with a low percentage of tumor bearers, as shown in TABLE 4, were relatively short-lived and resistant to the parasitic disease. Longevity or increased susceptibility to *Cysticercus* infestation contributed equally to a higher percentage of bearers of induced tumors. TABLE 5 shows a similar relationship for the F$_1$ and back-crossed hybrid between a short-lived susceptible and a longer-lived resistant strain of rats.

Among equally infected rats there was no difference in rates of occurrence of malignant disease in relatively susceptible and resistant strains of rats. TABLE 6[5] shows the percentage of induced sarcomas observed at each period of infestation from 10 to 22 months for 1027 rats from resistant strains and 675 rats from susceptible strains, each with a single parasite. The obviou similarity in rate of occurrence of tumors in equally infected rats of differen genetic constitution indicated that the carcinogenic property of a singl

TABLE 1
MEAN DURATION OF INFESTATION FOR BEARERS OF *Cysticercus* SARCOMA WITH SUCCESSIVE NUMBERS OF CYSTS

No. of cysts	No. of tumor bearers	Days/30 ± P. E.
1	411	18.82 ± 0.12
2	280	18.43 ± 0.15
3	181	17.68 ± 0.18
4	213	17.21 ± 0.14
5	174	17.25 ± 0.15
6	164	17.21 ± 0.16
7	137	16.08 ± 0.14
8	146	16.19 ± 0.17
9	104	16.05 ± 0.17
10	126	16.03 ± 0.15
11	91	15.81 ± 0.17
12	91	15.52 ± 0.18
13	80	14.91 ± 0.18
14	81	15.02 ± 0.18
15	87	14.78 ± 0.19
16	74	14.90 ± 0.18
17	78	14.64 ± 0.16
18	76	14.43 ± 0.16
19	57	14.36 ± 0.16
20	68	14.48 ± 0.17
21	72	14.46 ± 0.14
22	59	14.18 ± 0.21
23	45	14.28 ± 0.18
24	49	14.15 ± 0.22
25	35	14.90 ± 0.32
26–27	86	14.09 ± 0.15
28–29	94	13.99 ± 0.12
30–34	159	13.32 ± 0.10
35–39	99	13.50 ± 0.13
40–44	91	13.55 ± 0.12
45–49	53	12.84 ± 0.14
50–54	36	12.92 ± 0.20
55–59	28	12.68 ± 0.19
60–64	16	12.19 ± 0.31
65–over	28	12.86 ± 0.22
Total.......	3669	15.95 ± 0.04

aenia larva was independent of the maximum physiological or immuno-ogical influence exerted by the host in which it resided.

These observations seemed to indicate that the occurrence of neoplastic isease in the walls of *Cysticercus* cysts represented sudden permanent alter-tions in cells randomly exposed to an incitant not unlike the occurrence of omatic mutations in populations of cells randomly exposed to irradiation.

Following the discovery of the carcinogenic properties of the hydrocarbons n attempt was made to confirm and extend these observations. Data were aken on the occurrence of malignant disease in the walls of subcutaneously

TABLE 2

NUMBER OF INFESTED RATS WITH SUCCESSIVE NUMBERS OF *Cysticercus* CYSTS THAT SURVIVED THE MEAN PERIOD OF INFESTATION AND PERCENTAGE OF TUMOR BEARERS

No. of cysts	Total rats	Percentage of tumor bearers
1	724	31.49
2	343	39.07
3	206	46.60
4	189	53.44
5	142	63.38
6–9	377	65.78
9–12	303	66.67
12–15	172	69.19
15–18	190	74.21
18–21	159	74.82
21–24	124	83.06
24–27	77	84.42
27–30	117	82.90
30–33	73	71.23
33–36	70	80.00
36–39	48	81.25
39–42	40	85.00
42–45	32	87.50
45–48	30	80.00
48–51	21	85.71
51–54	16	81.25
54–57	17	58.82
57–60	16	68.75
60–63	9	66.67
63–66	14	50.00
66–69	4	75.00
69–72	1	100.00
72–75	2	50.00
75–78	7	85.71
78–81	4	25.00
81–84	1	100.00
84–87	2	100.00
87	4	25.00
Total.......	3534	58.11

injected paraffin cysts of carefully controlled size and accurately varie concentrations of the chemical carcinogen. For this purpose a 1 per cen solution of 3:4 benzpyrene[6] in warm paraffin wax was injected in the sub cutaneous tissues of rats in 1 to 12 discrete foci of 0.2 cc. volume. TABLE shows, for a series of 1261 induced neoplasms, that the average latent perio decreased from 193.7 days to 116.6 days as the number of injected foci wa increased from 1 to 8 and the dose of benzpyrene was increased coincidentall from 2 to 16 mg. A further increase to 12 foci or 24 mg. of benzpyrene di not further decrease the average latent period or reduce its variability. Th

TABLE 3

VERAGE WEIGHT IN GRAMS, PROPORTIONAL SIZE, AND NUMBER AND PERCENTAGE OF *Cysticercus* CYSTS AND TUMORS FOR EACH LOBE OF THE LIVER

Lobe of liver	Average weight in grams	Per cent of liver	Cysticercus cysts		Cysticercus tumors	
			Number	Per cent	Number	Per cent
eft lateral...........	2.83	31.21	567	27.96	1047	30.07
ight median.........	2.17	23.93	554	27.32	989	28.40
ranial right lateral...	1.52	16.79	361	17.80	497	14.27
eft median...........	1.01	11.14	226	11.14	429	12.32
audal right lateral...	0.72	7.97	169	8.33	261	7.50
entral caudate.......	0.41	4.48	91	4.49	133	3.82
Dorsal caudate........	0.40	4.47	60	2.96	126	3.62
otal................	9.07	100.00	2028	100.00	3482	100.00

TABLE 4

PERCENTAGE INFESTED, AVERAGE AGE AT DEATH, AND PERCENTAGE WITH *Cysticercus* SARCOMA FOR SEVERAL STRAINS OF RATS

Strain of rat	Percentage infested	Age at death, days/30 Mean ± P. E.	Percentage with *Cysticercus* sarcoma
Fischer-230.............	30.8	11.06 ± 0.13	25.0
August.................	21.6	14.03 ± 0.04	37.8
Marshall...............	37.9	13.5 ± 0.09	37.4
Zimmerman..............	61.8	11.9 ± 0.17	56.4
Fischer................	65.4	13.6 ± 0.10	60.0
Copenhagen.............	30.8	19.8 ± 0.13	62.7
A × C..................	31.5	21.7 ± 0.17	67.2
Fischer-344............	67.5	12.3 ± 0.10	67.2

TABLE 5

PERCENTAGE INFESTED, AVERAGE AGE AT DEATH, AND PERCENTAGE WITH *Cysticercus* SARCOMA, FOR A SUSCEPTIBLE AND A RESISTANT STRAIN OF RATS AND THEIR F_1 AND BACKCROSSED HYBRIDS

Strain of rat	Percentage infested	Age at death, days/30 Mean ± P. E.	Percentage with *Cysticercus* sarcoma
August.................	29.7	14.03 ± 0.04	37.8
Fischer-344............	66.1	13.6 ± 0.10	67.2
A × FF_1.................	32.7	16.2 ± 0.20	60.0
A × (A × F)............	29.4	16.4 ± 0.26	66.7
F × (A × F)............	38.3	15.1 ± 0.25	57.1

percentage of rats with induced sarcomas (TABLE 8) increased, however, from 80 to 100 as the number of foci was increased from 1 to 12. Similar observations were obtained following the injection of varying numbers of uniform foci of paraffin containing dibenzanthracene[6] and methylcholanthrene.[7]

TABLE 6

PERCENTAGE OF MALIGNANT CYSTS IN RATS WITH A SINGLE CYST FROM SUSCEPTIBLE AND RESISTANT STRAINS

Duration of infestation, days/30	Resistant strains, percentage malignant cysts	Susceptible strains, percentage malignant cysts
10–12	0.0	0.4
12–14	1.2	1.6
14–16	3.1	3.1
16–18	7.4	8.9
18–20	8.4	9.2
20–22	17.2	15.9
22	28.6	19.2
Total	65.9	58.3
Total No.	1027	675

TABLE 7

NUMBER OF TUMORS, MEAN NUMBER OF DAYS, AND STANDARD DEVIATION OF THE PERIOD FROM INJECTION TO OBSERVATION OF THE TUMORS FOR SEVERAL DOSES OF 3:4 BENZPYRENE

Dose (in mg.)	No. of foci	No. of tumors	Number of days	
			Mean ± P. E.	S. D. ± P. E.
2	1	103	193.74 ± 5.48	82.41 ± 3.87
4	2	229	163.91 ± 2.34	52.44 ± 1.65
6	3	282	150.74 ± 2.00	49.70 ± 1.41
8	4	175	142.94 ± 2.22	43.60 ± 1.57
10	5	157	149.71 ± 1.88	35.00 ± 1.33
12	6	189	146.80 ± 1.61	32.80 ± 1.14
16	8	49	116.63 ± 1.83	18.99 ± 1.29
24	12	77	117.99 ± 1.40	18.24 ± 0.99

These results confirmed the findings obtained from the observations on the occurrence of neoplastic changes in the walls of *Cysticercus* cysts. By the use of the hydrocarbons it was possible to extend these observations by varying the surface area of tissue exposed to a given quantity of the chemical carcinogen. Thus[8] 0.2 cc. of a 1 per cent solution of benzpyrene was injected in a single area forming a cyst containing 2 mg. of active carcinogen, or in

TABLE 8
PERCENTAGE OF RATS WITH INDUCED SARCOMAS FOR EACH NUMBER OF FOCI OF BENZPYRENE

No. of foci	No. of rats	Percentage of tumor rats
1	46	80.4
2	78	82.0
3	53	84.9
4	76	85.5
5	49	91.8
6	74	82.4
8	16	87.5
12	17	100.0
Total...	409	84.7

2 foci of 0.1 cc. volume each containing 1 mg. of benzpyrene, or in 4 foci of 0.05 cc. volume, each cyst containing 0.5 mg. of benzpyrene. Four mg. of benzpyrene was likewise distributed in 2, 4, or 8 foci. By this method the surface area of tissue exposed to 2 mg. of benzpyrene was varied from approximately 186 sq. mm., to 292 sq. mm. and 412 sq. mm. Likewise, the surface area of tissue exposed to 4 mg. of benzpyrene was increased from 372 sq. mm. to 584 sq. mm., and 824 sq. mm. Weekly observations on the occurrence of sarcomas in the foci of different volume showed (TABLE 9) that tumors occurred in rats with 2 mg. of benzpyrene in a single focus in an average of 193.7 days. First tumors appeared in foci of 0.1 cc. volume in an average of 197.6 days and, when 2 mg. of benzpyrene was injected in 4 foci, the first tumors appeared in an average of 222.9 days. In other words, as the surface area of tissue exposed to 2 mg. of benzpyrene was increased from 186 sq. mm. to 412 sq. mm., or more than doubled, the average latent period preceding the onset of malignant disease was increased by 29 ± 8.8 days. For 4 mg.

TABLE 9
AVERAGE LATENT PERIOD FOR BENZPYRENE SARCOMAS INDUCED IN FOCI OF DIFFERENT VOLUME

Dose in mg.	Foci of 0.2 cc. volume		Foci of 0.1 cc. volume		Foci of 0.05 cc. volume	
	No. tumors	Latent period in days Mean ± P. E.	No. tumors	Latent period in days Mean ± P. E.	No. tumors	Latent period in days Mean ± P. E.
2	103	193.7 ± 5.5	111	197.6 ± 5.1	93	222.9 ± 6.9
4	143	157.6 ± 2.9	105	176.3 ± 4.3	95	198.6 ± 5.5
6	129	140.7 ± 3.4	74	155.4 ± 3.4		

of benzpyrene the average latent period preceding the observation of th first localized neoplasms increased from 157.6 to 176.3 and 198.6 days, respectively, as the foci decreased in volume and the surface area of tissue expose to 4 mg. of benzpyrene increased from 372 sq. mm. to 824 sq. mm. Th average latent period was significantly increased rather than decreased, a would be expected if neoplastic changes occurred at random in totipoten cells exposed to a uniform intensity of incitant.

Furthermore, the percentage of rats that developed neoplasms was no increased by exposure of larger areas of tissue to a given quantity of benzpyrene. TABLE 10 shows that among rats injected with 2 mg. of benzpyren

TABLE 10
PERCENTAGE OF RATS WITH BENZPYRENE-INDUCED SARCOMA AMONG THE RATS INJECTED WITH FOCI OF DIFFERENT VOLUME

Dose in mg.	Foci of 0.2 cc. volume		Foci of 0.1 cc. volume		Foci of 0.05 cc. volum	
	No. of rats	% tumor rats	No. of rats	% tumor rats	No. of rats	% tumor rats
2	146	70.6	145	76.6	138	67.4
4	176	81.2	138	76.1	132	72.0

71, 77, and 67 per cent, respectively, developed neoplasms in the groups wit 1, 2, and 4 foci. For 4 mg. of benzpyrene 81, 76, and 72 per cent, respectively, developed malignant disease among those injected in 2, 4, and 8 foci Since 2 mg. of benzpyrene injected in a single focus was more effective in th induction of malignant disease than when it was distributed in smaller foci the mass of the carcinogenic material must have played a determining role.

The importance of variations in the intensity of the local incitant wa shown[9] when concentrations of 1 per cent, 0.25 per cent, and 0.10 per cen benzpyrene were injected in foci of equal volume. TABLE 11 shows that, fo

TABLE 11
PERCENTAGE OF INDUCED SARCOMAS FOR DIFFERENT CONCENTRATIONS OF BENZPYRENE

No. foci per rat	Dose in mg.	No. foci	Percent-age of tumor foci	Dose in mg.	No. foci	Percent-age of tumor foci	Dose in mg.	No. foci	Percent-age of tumor foc
1	2	146	70.6	0.5	58	43.1	0.2	52	13.5
2	4	324	70.7	1.0	121	36.4	0.4	94	13.8
3	6	438	64.4	1.5	197	35.5	0.6	157	15.3
4	8	291	60.1	2.0	253	29.2	0.8	190	13.7
5	10	241	65.2	2.5	260	21.9	1.0	179	13.4
6	12	399	47.4	3.0	305	33.1	1.2	213	16.0

xample, 70.6 per cent of 146 rats with 2 mg. of benzpyrene, 43.1 per cent f 58 rats with 0.5 mg. of benzpyrene, and 13.5 per cent of 52 rats with .2 mg. of benzpyrene developed sarcomas at the sites of injection. All eceived injections of 0.2 cc. of paraffin with consequent exposure of an equal umber of host cells to the incitant. The average latent periods were, respec-ively, 193.7, 267.4, and 289.3 days, as shown in TABLE 12. These experi-nents showed that an increase in the concentration of the locally applied arcinogenic chemical significantly decreased the average latent period that receded the occurrence of malignant disease and significantly increased the robability of its occurrence. These data suggest that the probability of the occurrence of malignant disease and the time required to induce it are etermined by the extent of some specific injury to the cells.

TABLE 12
AVERAGE LATENT PERIOD IN DAYS FOR DIFFERENT CONCENTRATIONS OF BENZPYRENE FOR EACH NUMBER OF FOCI OF EQUAL VOLUME

No. foci per rat	Dose in mg.	No. of tumors	Latent period Mean ± P. E.	Dose in mg.	No. of tumors	Latent period Mean ± P. E.	Dose in mg.	No. of tumors	Latent period Mean ± P. E.
1	2	103	193.7 ± 5.5	0.5	25	267.4 ± 13.2	0.2	7	289.3 ± 34.7
2	4	229	163.9 ± 2.3	1.0	44	244.8 ± 10.7	0.4	13	304.2 ± 28.1
3	6	282	150.7 ± 2.0	1.5	70	267.6 ± 8.6	0.6	24	345.0 ± 13.9
4	8	175	142.9 ± 2.2	2.0	74	251.4 ± 7.1	0.8	26	280.4 ± 14.6
5	10	157	149.7 ± 1.9	2.5	57	242.0 ± 8.3	1.0	24	287.9 ± 14.6
6	12	189	146.8 ± 1.6	3.0	101	271.1 ± 6.9	1.2	34	348.2 ± 14.3
Total..		1135	155.2 ± 1.0		371	258.7 ± 3.5		128	314.8 ± 7.2

Ample proof of the importance of qualitative differences in the compo-ition of the chemical carcinogens has been provided by other investigators. ABLE 13 shows the average latent period observed for the occurrence of ubcutaneous sarcomas in rats following the injection of 4 mg. of a 1 per cent olution of 4 of the more potent carcinogens. At this dose level no difference

TABLE 13
AVERAGE LATENT PERIOD IN DAYS FOR HYDROCARBON-INDUCED SUBCUTANEOUS SARCOMAS IN RATS

Chemical	Dose in mg.	No. of tumors	Days ± P. E.
Methylcholantrene..................	4	56	161.8 ± 2.8
3:4-Benzpyrene.....................	4	229	163.9 ± 2.3
10 Methyl 1,2-benzanthracene.......	4	52	200.9 ± 2.7
1:2:5:6-Dibenzanthracene...........	4	16	330.0 ± 12.9

was observed between methylcholanthrene and 3:4-benzpyrene in the ave age latent period that preceded the occurrence of the induced sarcoma However, for 1:2:5:6-dibenzanthracene the average latent period wa significantly longer than for 10-methyl 1:2-benzanthracene, and both wer significantly longer than for methylcholanthrene or benzpyrene.

TABLE 14 summarizes the results obtained from injections of each of th monomethyl derivatives of 1,2-benzanthracene. Each compound wa injected subcutaneously in a 1 per cent solution of paraffin wax in 2 foo of 0.2 cc. volume in each of 60 Fischer line 344 rats. In this experiment uniform dose of each compound was exposed to an equal surface area tissue of the same highly inbred strain of rat. The difference in response i

TABLE 14
RELATIVE CARCINOGENIC ACTIVITY OF THE MONOMETHYL DERIVATIVES OF 1,2-BENZANTHRACENE

Chemical	Dose in mg.	No. of rats	Percentage of tumor rats	No. tumors	Average latent period ± P. E. in day
10-Methyl 1,2-B-A....	4	60	93.3	97	200.9 ± 2.7
4-Methyl............	4	58	70.7	58	234.8 ± 4.1
5-Methyl............	4	59	61.0	41	265.2 ± 8.7
9-Methyl............	4	61	52.5	39	292.2 ± 10.7
7-Methyl............	4	57	5.3	3	396
6-Methyl............	4	59	5.1	3	413
4′-Methyl...........	4	60	5.0	3	495
3-Methyl............	4	55	3.6	2	417
3′-Methyl...........	4	54	0.0		
2′-Methyl...........	4	61	0.0		
8-Methyl............	4	58	0.0		
1′-Methyl...........	4	60	?	?	
1,2-Benzanthracene...	4	56	1.8	1	399
Total		758		247	

obvious. Four of the compounds, 10-methyl-, 9-methyl-, 4-methyl-, an 5-methyl- proved to be highly carcinogenic. Four other compounds, namel 4′-methyl-, 3-methyl-, 6-methyl-, and 7-methyl- were found to be weakl carcinogenic and 3 or probably 4 (tests for 1′-methyl- are incomplete) wer found to be completely negative.

These are some of the localized differences in response of tissues of simila genetic constitution that pose problems for the genetic concept of the origi of cancer. In all of the above-mentioned experiments malignant diseas arose as an extremely localized response to a long-continued unfavorabl environment. Other methods for the experimental induction of malignan disease pose different problems. For instance, hyperestrinism in rats pr duces a generalized hyperplasia of the ducts of the mammary gland and, the stimulation is continuous for one half or more of the life span of the ra many areas of neoplastic disease arise simultaneously. The average later eriod may be decreased and the number of independent neoplasms increased n animals of the same genetic constitution by the addition of fat[10] or trypto- han[11] to the diet. Likewise chronic poisoning with acetylaminofluorene roduces a cirrhosis of the liver, followed by areas of hyperplasia of the bile ucts and liver cords with simultaneous neoplastic transformation of many reas. The average latent period may be considerably shortened by the ddition of fat, protein, tryptophan, or indole to the diet. These experi- ents suggest that neoplasia arises as a localized biological response of the arget organ or tissue to alteration or destruction of some mechanism in the ell that results from specific chemical reaction under local physiological ontrol. The fact that an increase in dose of an effective carcinogen increases he probability of the occurrence of malignant disease to an almost certainty nd reduces the average latent period to a limit below which further increases n dosage are ineffective suggests that the occurrence of malignant disease esults from localized breakdown of some system that is common to all omatic cells.

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THE AGING PROCESS AND CANCEROGENESIS

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The Aging Process

The solid curve of FIGURE 1 represents the age-specific death rate of th male population of New York, N. Y., in 1950 as a function of age. The ord nate represents, on a logarithmic scale, the fraction of the individuals of given age dying per year and the abscissa the age. It will be seen tha beyond age 30 the curve approximates a straight line, the equation of whic is of the form

$$r = r_o e^{\alpha t}$$

where r_o = mortality rate at age 0, from all causes
e = base of natural logarithms
α = slope of straight line
t = age in years

This was first pointed out by Gompertz[1] in 1825, and the relation is no generally called the Gompertz function. From the slope of this curve may be calculated that the mortality rate doubles about every 7.2 year It has been found that, whereas the initial portion of the curve (before ag 30) varies greatly from one country to another and in the same countr from decade to decade, the slope of the straight portion remains approx mately the same. A general discussion of these curves has been given b Jones[2] and need not be repeated here. It has been found, also, that simila curves apply to animal populations, as shown in FIGURE 2 for mice and ra (the curve for mice was constructed from survival data of Sacher[3] and tha for rats from observations of Simms and Berg[4]).

The dashed curve in FIGURE 1 was obtained by subtracting from th over-all mortality rate data those deaths caused by accident, homicide, suicide. The exclusion of deaths from these causes (which account for considerable proportion of the total deaths before age 30) has the effect, smoothing out the mortality rate curve at the early ages as shown.

It may be surmised readily that the increase in mortality rate in th straight line portion of the curves is due to an aging process that is comm to man, mouse, and rat. The doubling time for the particular mouse colo of FIGURE 2 is 90 days; that for man (FIGURE 1) is 7.2 years, or about times longer than that of mouse. This is also approximately the ratio the life spans of man and mouse.* Therefore, the life span determines t rate at which animals age after reaching adulthood, which in a general w is obvious a priori.

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* It should be noted that the slope of the Gompertz curve for mice, plotted from availa data in the literature, varies considerably according to strain, sex, laboratory conditio and numerous other factors. Also, the number of animals is usually too small to prov good curves.

The life span is determined by the genetic constitution of the germ cells. An egg and a sperm that produce a mouse must be responsible for its short ife span compared to that of man. Geneticists have found that the over-all spontaneous mutation rate for germ cells is roughly—very roughly—nversely proportional to the life span of the species. This means that the ccumulated genetic changes (that is, in the germ cells) per generation are approximately the same in short-lived and long-lived animals. The genetic material is considered to be the same in somatic cells as in the germ cells from which they were derived; therefore, the somatic mutation rate per unit of time may be expected to be higher in short-lived animals than in long-lived ones. Thus, we may assume that the genetic system in all cells of the human body is more stable than that of the cells of a mouse, generally in the ratio of the life spans. "More stable" is used here in the sense that the system is less apt to undergo spontaneous mutations *in a given time interval.*

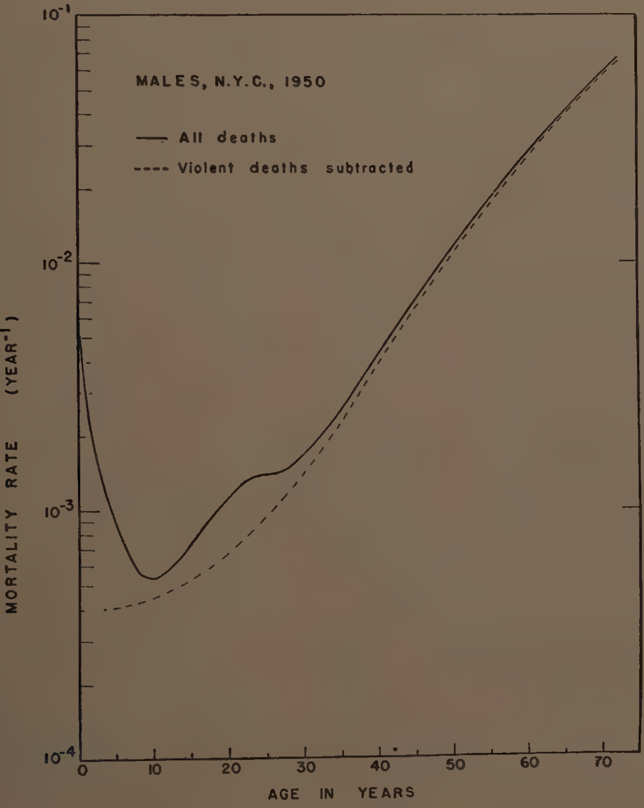

Figure 1. The solid curve represents the mortality rate of the male population of New York, N. Y., in 1950 as a function of age. The dashed curve was obtained by subtracting violent deaths, that is, those caused by accident, homicide, or suicide.

The system consists of the number of genes, geometric pattern, and require properties appropriate to the animal under consideration. It is not necessary to assume that the spontaneous mutation rate is the same in all somati cells as in the germ cells. Therefore, the mutation rate in skin cells migh

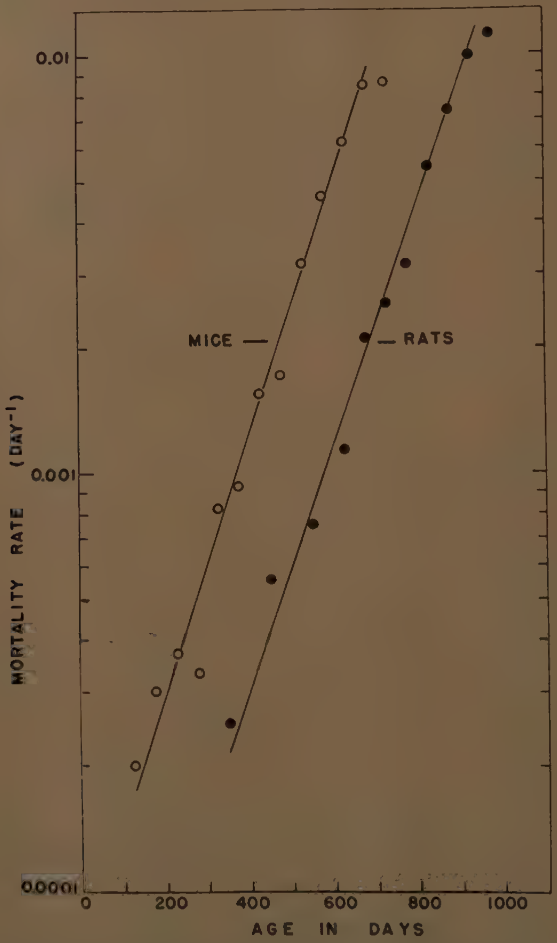

FIGURE 2. Age-specific mortality rates for mice and rats. The data for mice wer derived from a survival curve for CF No. 1 female mice published by Sacher.[3] The curv for rats is based on longevity data for Sprague-Dawley male rats published by Simms an Berg.[4]

be ten times that of the germ cells in mouse. However, if there is a differenc in mutation rate from cell type to cell type in the same animal, an approximately proportionate difference should be present among the cell types o an animal of a different species. For the present purpose the importan point is that the average spontaneous mutation rate of the somatic cells o a short-lived species be higher than that of the somatic cells of a long-lived species and approximately in the inverse ratio of the life spans. As mentioned above, this is roughly the case for spontaneous mutations in the germ cells—the only ones that can be studied readily—and it is reasonable to assume that the same general relation holds for spontaneous mutations in somatic cells. If this is the case, aging may be attributed to the accumulation of spontaneous somatic mutations in all body cells, but not necessarily in the same proportions in all organs. Thus, we may visualize the following simple process by which the life span is controlled in nature: the constitution of the genetic system in the germ cells determines the inherent stability of the genetic system of the body cells in a given species. The spontaneous mutation rate is determined by the stability of the genetic system, being lower the higher the inherent stability. The rate of aging is determined by the spontaneous (somatic) mutation rate. Aging increases the mortality rate according to the Gompertz function and sets a limit to the life span.

We may now inquire how spontaneous somatic mutations bring about aging. To do this we shall define vitality as the reciprocal of age-specific mortality rate. Thus, according to FIGURE 1, the mortality rate at age 35 is 0.0023 (2.3 per 1000) and at age 50 it is 0.010 (10 per 1000) per year. Therefore, according to this notation the vitality of a man of 50 is $\frac{2.3}{10} = 23$ per cent of the vitality of a man of 35. The reciprocal of the age-specific mortality curve of FIGURE 1 then represents the vitality of the New York, N. Y., male population of 1950 as a function of age, ignoring for the present discussion the high mortality rate in the first few years of life. This is shown in FIGURE 3. By inspection it may be surmised that this is a "multiple-hit" or a "multiple-target" type of curve. Therefore, loss of vitality with age may be attributed to loss of normally functioning cells, the deterioration in function of the cell being brought about by multiple hits or hits in a number of targets in the cell. It is obvious that the loss of vitality cannot be attributed to actual loss of cells, because the number of cells at age 60 would have to be about 2.5 per cent of the number at age 20. It is known that in old people the cell population in a tissue is not very different from that in young subjects. Hence we must assume that the cell population is affected in function rather than in number.

It is a tenet of genetics that the vast majority of germ-cell mutations is deleterious to the ensuing organism and its descendants. If we assume that, in general, somatic mutations are deleterious to the cell and its descendants, in the sense that the function of the cell is impaired, we can account for the shape of the curve on the basis of multiple gene mutations. At this point we should decide whether some particular genes or a number of genes at random are involved in the reaction. This determines whether we are dealing with a multiple-target or a multiple-hit phenomenon. Actually, the curve can be represented closely by the equation for either process, but perhaps it is simpler to visualize a multiple-hit process. The solid curve in FIGURE 3 has been plotted from the equation for a four-hit process, and the circles represent the reciprocals of the age-specific mortality curve of FIG-

URE 1, normalized to 1.0 at age zero for convenience. It should be note in this connection that extending the curve to zero age is not justified, bu is satisfactory for the present discussion. It will be seen that the fit is very good. Therefore, we may conclude that the increase in mortality rate wit age (in the male population of New York in 1950) may be accounted for o

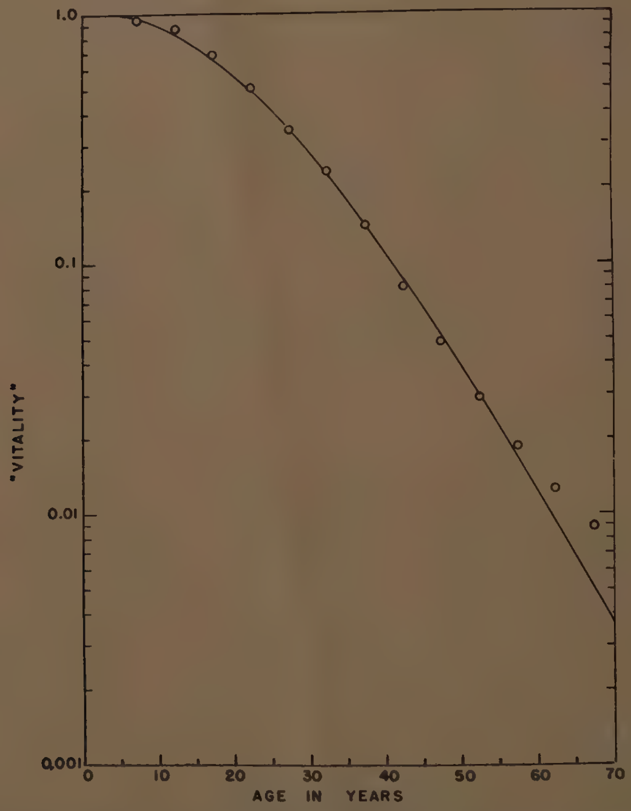

FIGURE 3. "Vitality" as a function of age. The circles represent the reciprocals the age-specific mortality rate data shown in the dashed curve of FIGURE 1. The soli curve has been plotted from the equation for a four-hit process.

the basis of a four-hit random process. Tentatively we may postulate tha each "hit" corresponds to the spontaneous somatic mutation of one gene i the cell. Then it would follow that, on the average, four genes at rando (that is, any four genes) in each cell must be mutated before its function impaired sufficiently to contribute to the aging process.

The equation for the curve of FIGURE 3 is of the form

$$\frac{N}{N_0} = e^{-\alpha t}\left[1 + \alpha t + \frac{(\alpha t)^2}{2} + \frac{(\alpha t)^3}{6}\right]$$

where $\frac{N^*}{N_o}$ = fraction of cells that have not undergone 4 gene mutations at a given age; e = base of natural logarithms; α = fraction of cells in which one gene is mutated per year (or probability of one gene mutation occurring per cell per year); and t = age in years. It is obvious that the probability of one gene mutation occurring in one cell per unit time is proportional to the number of genes in the cell. Therefore,

$$\alpha = nm$$

in which n = the number of genes per cell and m = the probability of one gene mutating per year. Geneticists express this in terms of one generation rather than per year, so that if M is the gene mutation rate per generation of 30 years in man,

$$m = \frac{M}{30}$$

Therefore,

$$\alpha = n\frac{M}{30}$$

The value of α derived from the curve of FIGURE 3 is 0.162. The haploid gene number in man has been estimated to be between 5000 and 100,000, as quoted by Neel and Schull.[5] Assuming that it is 20,000, the number in somatic (diploid) cells, with which we are concerned here, is 4×10^4. Therefore,

$$0.162 = 4 \times 10^4 \frac{M}{30}$$

or

$$M = 12 \times 10^{-5}$$

Accordingly, the gene mutation rate in somatic cells in man is 12×10^{-5} per generation of 30 years, on the basis of a four-hit curve derived from mortality statistics and the assumption that the increase in the age-specific death rate with age is due to somatic mutations. The gene mutation rate in germ cells in man is estimated by geneticists to be 10^{-5} per generation. If we accept these two values, it may be concluded that in man the spontaneous gene mutation rate in somatic cells is approximately 12 times that in germ cells. It is of interest to note in this connection that in *Drosophila* the number of abnormalities in the offspring produced by a given dose of X rays is about four times greater when the experimental conditions are such that the effect of radiation is on spermatozoa rather than on spermatogonia.[6] This may well be due to chromosome damage rather than additiona gene mutations produced in spermatozoa, since spermatogonia with badl damaged chromosomes would not be expected to reach the spermatozoa stage; therefore, those that do reach this stage are the ones in which th chromosomes were not badly damaged at the start. At any rate, chromosome damage in somatic cells does occur spontaneously. Hence, it may b concluded that the "mutation" rate derived from the age-specific mortalit curve includes chromosome damage as well as gene mutation. Then, if th factor of 12 noted above is real, it would be concluded that aging is du largely to the accumulation of chromosome damage in the somatic cells o the body. This has been suggested by some geneticists, notably Muller. At the present time it is really impossible to make any definite statemen about this, because the uncertainty in the available genetic data for man i too great. However, it is reasonable to suppose that both gene mutation and chromosome damage play a part in the aging process. In this case i would be more appropriate to speak of a "cell impairment rate" due t nuclear damage rather than a "somatic mutation rate" in connection wit the aging process.

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* It should be noted that the ratio $\frac{N}{N_o}$ is applicable to any age, including the period of rapid growth. In other words, the thing that counts is the number of cells per unit volume rather than the total number of cells in the body or an organ.

The multiple hit concept was introduced to take into account the rathe slow increase in age-specific death rate from approximately age 5 to 30. In so doing it was assumed that the smooth curve of FIGURE 1 truly represents the aging process. It may well be that the high mortality in this period i due to other causes. For one thing, in the case of mice and rats (FIGURE 2) the existence of a plateau in mortality rate at early age is questionable, but this may be due to the paucity of data.* In the case of man, from about age 30 to 60 or so, the data fit a straight line very well. It is important to note in this connection that the data are derived from the vital statistics of millions of individuals and, therefore, are very reliable. If it is assumed that this straight line represents the aging process per se, we are dealing with a single-hit process. The equation for the "vitality" curve (straight line) derived from FIGURE 1 is

$$\frac{N}{N_o} = e^{-0.0963t}$$

in which 0.0963 is the value of α in the notation used previously. Hence

$$0.0963 = 4 \times 10^4 \frac{M}{30}$$

and

$$M = 7.2 \times 10^{-5}$$

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* It would be of great interest to see what the curve looks like in the case of a larg colony of animals reared under germfree conditions. Incidentally, it is known that unde these conditions there is some prolongation of life, but not a marked one, which lend support to the idea that the life span is controlled by the inherent stability of the geneti system.

Therefore, if this represents the gene mutation rate in somatic cells in man, it is closer to the gene mutation rate in germ cells estimated by geneticists (10^{-5} per generation). In view of the assumptions made and the uncertainties in the genetic data, all that can be said is that the single-hit hypothesis yields a value of the gene mutation rate in man of the same order of magnitude as that estimated by geneticists.*

It should be noted that the value of α can be obtained with considerable accuracy when the one-hit process is assumed. If it is further assumed that aging is due to somatic gene mutations, α represents the probability of a gene mutation occurring per cell per unit time. This does not involve the number of genes in a cell, which is difficult even to estimate, but nevertheless serves as a measure of the stability of the genetic system of the cell with respect to time. The values of α for mouse and rat, derived from the curves of FIGURE 2, are 7.7×10^{-3} days^{-1} and 6.3×10^{-3} days^{-1}, respectively. The one for man is 0.0963 years^{-1} or 2.64×10^{-4} days^{-1}. The stability of the genetic system is inversely proportional to α. The ratio of the α's for mouse and man is 29, which is approximately the ratio of the respective life spans. For rat and man the ratio is 24, which is in agreement with the known fact that the life span of the rat is somewhat longer than that of the mouse. As pointed out earlier, the Gompertz curves for groups of animals in different laboratories are apt to vary considerably, and the statistical reliability is low when small groups are used. Therefore, these ratios have only a qualitative significance.

In summary, it may be said that aging in the straight-line portion of the age-specific mortality curve may be attributed to a one-hit process that impairs the function of somatic cells at a rate inversely proportional to the life span. Defining vitality (in the sense of ability to survive rather than ability to do work) as the reciprocal of age-specific mortality rate, it is found that vitality at any given age is proportional to the fraction of cells $\left(\frac{N}{N_o}\right)$ that have not been "hit" at that age. The exponential decrease in vitality with age indicates that the impairment in function of the cell brought about by the hit is permanent. Since gene mutations are "permanent" and may well cause malfunctioning of a somatic cell, it is not unreasonable to assume that the hit represents a spontaneous gene mutation. Permanent chromosome damage could also be implicated. However, in the present state of knowledge of the aging process, it is not possible to exclude mechanisms that have nothing to do with spontaneous mutations and/or chromosome damage. Perhaps the strongest inferential support for the idea that somatic mutations are fundamentally responsible for the aging process (represented by an exponential increase in mortality rate with age) comes from the fact that the genetic constitution of an egg and a sperm determines what kind of animal (species) will result from their union, and the maximum life span for that animal is a characteristic of the species. It is known that the over-all spontaneous gene mutation rate in germ cells is roughly inversely proportional

* A more detailed analysis of the whole age-specific mortality curve for man, in terms of the mutation theory, will be made in a subsequent publication.

to the life span for different species, and it may be expected that the somati mutation rate follows the same general relation. Then the assumption tha the increase in mortality rate with age is due to the accumulation of spontaneous somatic mutations provides a simple and plausible mechanism fo the control of the life span of a species by the initial genetic constitution o the egg and sperm of that species. Obviously, much more information i needed to confirm or disprove the assumption.

Cancerogenesis

The sequence of events in the development of cancer of the skin may b visualized most readily from observations on radiation-induced neoplasms Before the very late sequelae of radiation damage were fully realized many patients were treated with low-voltage X rays for cosmetic purposes (fo example, removal of superfluous hair) and for a variety of dermatologica conditions. Single treatments and, more often, repeated treatments were given with doses that produced a definite but not necessarily a marked ski reaction during the course of treatment. In general, after a few weeks o a few months the skin regained its "normal" appearance (including, sometimes, the diseased condition for which the treatment had been given). In a considerable proportion of the cases new changes in the appearance of the skin became evident after a number of years and, in some cases, neoplasms developed still later (usually 15 to 20 years after the initial treatment). In the case of the early radiologists and technicians who exposed their hands to X rays daily, skin changes were produced gradually and may not have been apparent for a number of years. In many instances cancer of the skin developed later, the latent period being somewhat related to the degree of exposure.

As far as the present discussion is concerned, the dermatological patients and the professionally exposed persons serve the same purpose. In both, skin cancer developed after a period of many years in small regions of the irradiated areas that had shown definitely visible changes for a considerable time. The most interesting thing is that tissue specimens taken from these regions for histological study indicate a gradual transition from normal to neoplastic. That is, in the same irradiated area there are regions in which cancer is or is not present, but there are also regions that the pathologist is able to classify as definitely precancerous. Accordingly, there must be a gradual change in the irradiated skin (over a period of years) up to the time that a neoplasm is definitely established. Since it is known that ionizing radiation is a powerful mutagenic agent, it is natural to suspect that somatic mutations may be responsible for the initiation of the cancerous process. In this must be included the skin injury that appeared some years after the treatment and the precancerous state. We shall consider first a possible mechanism for the production of a cancer cell by radiation.

The most outstanding characteristic of a cancer cell is its ability to divide indefinitely. In normal tissues in which cell multiplication occurs naturally (for example, the skin) to take care of wear and tear and to repair damage, cel division is carefully controlled. For instance, if the skin is cut, cell divisio s greatly accelerated, but returns to normal as soon as the damage has been repaired. To account for this we may postulate that each cell produces an "antiproliferating agent" (APA) which, when present in a certain concentration, prevents the cell from dividing. When the skin is cut, the concentration of this agent at the boundaries of the cut decreases and the neighboring cells begin to divide. When healing is complete the concentration returns to normal. The control of cell division is, therefore, a collective one; that is, all the cells in a certain volume of tissue contribute to it.

It is not unreasonable to assume that the production of the APA is due to a particular gene in the cell, or, more likely, to the two genes of an allelic pair. Therefore, if suitable mutations of these genes occur, the cell no longer produces the agent. The cell then acquires the potentiality of indefinite division; that is, it becomes a cancer cell. However, it does not follow that a tumor will develop, because normal cells surrounding it are able to maintain a sufficient concentration of the agent around the mutated cell. Hence, in order for the tumor to develop, a certain number of the normal cells around the mutated cell must not function properly or, at any rate, diffusion of the APA from normal cells to the mutated cell must be blocked or interrupted. If this local situation exists for a sufficient length of time, uncontrolled division starts. Once the number of cancer cells has reached a certain value (that is, the tumor has reached a certain size) the concentration of the APA at the boundary will always be subnormal and the tumor keeps on growing. It will be seen, therefore, that the establishment of a tumor depends on the accidental juxtaposition of a cancer cell (which has arisen from a mutated normal cell) and a region in which the concentration of APA is abnormally low for one reason or another.

In view of the sequence of events in the development of radiation-induced cancer of the skin, it may be concluded that the skin injury, which develops slowly over a period of years, provides the required conditions (low concentration of APA according to the mechanism just outlined) for the growth of a tumor. The absence of a marked acute skin reaction and the long "latent" period suggest that the late skin damage may be attributed to somatic mutations produced by the radiation in the germinal layer of the epidermis. That is, after the acute reaction, there remained cells that had been mutated in such a way that they were able to survive; their function, however, had been impaired. A certain amount of time was required for the phenotypic manifestation of the genotypic change (which occurred at the time of irradiation), and more time was required for the injury to develop through competitive processes between mutated and nonmutated cells. Finally, the situation was aggravated by the accumulation of spontaneous somatic mutations with increasing age, as already postulated. Of course, additional time was needed for the initial cancer cell to produce a detectable tumor by repeated divisions.

The question arises now as to whether the initial cancer cell (involving mutations in specific loci) was produced by the radiation at the time of treatment or was produced later by spontaneous mutations, when the terrain was more favorable for the establishment of a tumor. Obviously, the question cannot be answered categorically. It is known that cancer cells can be dormant for many years before a recurrence or metastasis of a tumo occurs. On the other hand, it is difficult to see how a cancer cell in skin tha is constantly being renewed could remain dormant for as many as 45 years— the longest period in which a skin cancer is known to have developed in th irradiated area of a patient originally treated for an internal cancer. On the basis of the mechanism postulated above, it may be possible that a cance cell produced by the radiation could not establish a tumor because the terrain was not favorable (that is, the concentration of APA was not low enough at the time and, in due time, it died. Thus, a neoplasm that appears very late may well be attributed to the gradual development of the propitious terrain in the irradiated area and a subsequent mutation of a special kind occurring spontaneously in one of the cells in this terrain.

The situation is somewhat different in the case of the hands of radiologists in which the damage is due to chronic exposure over a period of many years Here the favorable terrain results from the accumulation of more or less permanent radiation damage and the probability of occurrence of a cancer cell therein is increased by the continued exposure to radiation.

The infrequency of cancer of the skin in patients who receive large skin doses in the treatment of deep-seated tumors requires elucidation. In such cases the irradiation is usually completed in a period of a few weeks. The considerable acute skin damage that is produced is due in large measure to killing of cells. Replacement of dead cells is a normal function of the skin therefore, if the damage is not too great, *apparently* complete restoration takes place. There is nevertheless some permanent damage that may cause trouble later. Paradoxically, the difference between the dermatological cases mentioned earlier and patients receiving deep X-ray therapy may be attributed to the larger doses used in these cases, which *kill* many cells.[*] These are later replaced by normal or nearly normal cells. There are, of course, other differences, such as the age of the patients and the survival time. Most of the dermatological cases that developed skin cancer later were treated in their teens for minor skin ailments. On the other hand, patients treated for internal cancers are usually much older and die of the disease in a few years—before skin cancer is able to develop. The depth of tissue receiving a large dose, which is much greater in the case of deep X-ray therapy, should be considered also. Damage to the vascular bed may well interfere with the development of cancer, for instance, by restricting the food supply. Further consideration of these quantitative differences would take us too far afield. The point of chief interest at the moment is that according to the proposed mechanism, cancer develops in an irradiated skin area because many of the cells therein are descendants of cells originally mutated by the radiation and function subnormally. In some regions groups of these cells provide a terrain favorable to the development of cancer when, by a particular kind of mutation (spontaneous or otherwise), one is produced in such a region.

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[*] With small doses a few cells may be killed by the production of lethal gene mutations the cells dying when they attempt to divide or after they have gone through a few divisions With larger doses cells may die also because of extensive chromosome damage. With stil larger doses direct interference with metabolic process at the chemical level (for example destruction of enzymes) plays an important part in the killing of cells. Obviously th cells that have been killed can cause no trouble later, provided they are replaced by norma cells. The complications arise from the fact that at least some of these cells have bee mutated so that they and their descendants function subnormally.

If this mechanism for the production of skin cancer by radiation is accepted, it is not difficult to extend it in broad outline to all primary cancers of the skin. The extension to neoplasms produced by chemical carcinogens is obvious, especially when the compound is known to be mutagenic. Some are not mutagenic* and, according to the cancerogenic mechanism proposed here, they need not be. All that is necessary is that they be able to induce, in a certain volume of normal tissue, a favorable terrain for the multiplication of a cancer cell already there or produced later by the appropriate spontaneous mutation of a normal cell. In animals chemical carcinogens applied to the skin do produce hyperplasia, and a precancerous state is recognizable before the neoplasm is definitely established. As mentioned earlier, a precancerous state exists in localized skin areas of the hands of radiologists who later develop skin cancer from overexposure to ionizing radiation. The presence of hyperplasia is significant in connection with the hypothesis that a low concentration of APA is required to start the establishment of a tumor. The presence of rapidly dividing cells in a normal tissue seems to favor tumor development in general. Thus, skin tumor formation by chemical carcinogens is enhanced by wound healing. Radiation-induced cancers of the skin in man occur mostly in regions in which the integrity of the skin is difficult to maintain; that is, repair is constantly attempted, but is not quite successful. Perhaps because the skin is thinner and more easily damaged mechanically, practically all neoplastic lesions in the hands of radiologists occur on the outer aspect of the hands. It should be noted, however, that this side is also the one that receives the highest radiation dose. On the other hand, spontaneous skin cancers occur also mostly in the same skin area (outer aspect of the hands), so that trauma resulting in skin breakage must play a considerable part. Open wounds are less apt to occur in the palms of the hands where the skin is thicker and the metabolically active cells are better protected. In spontaneous skin cancers, the effect of sunlight (particularly ultraviolet) must also be taken into account.

It is a matter of common observation that, with advancing age, the skin becomes devitalized, drier, less pliable, and more apt to crack. Keratoses and other manifestations of local physiological malfunction are common. These grow progressively worse or more numerous. Since the deterioration is permanent, it is evident that there must be a deterioration of the cells that are responsible for the maintenance and renewal of the surface layers of the skin. The process may be considered to be a special (regional) case of the aging process already discussed. As pointed out under *The Aging Process*, somatic mutations may be assumed to cause impairment of function of cells. In the case of the skin a mutated cell in the germinal layer* may be considered inferior to a normal one, and its descendants (the more differentiated skin cells) are also defective. Lethal mutations in skin cells are of no importance, at least up to a certain point, since new cells to take their places can be produced by the remaining ones. While the skin as a whole shows deterioration with advancing age, some local regions manifest greater damage. This may be accounted for by the randomness of the mutation process, which occasionally results in the grouping of mutated cells in clusters. Thus, according to the mechanism proposed here, spontaneous mutations give rise to local skin imperfections. Some of these provide a terrain suitable for the development of cancer. If and when a normal cell in this region undergoes mutations of the special kind that transform it into a cancer cell a neoplasm may become established. Obviously, the local conditions of the tissue must also be suitable for the cancer cell to perform the necessary metabolic processes for division and multiplication.

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* It might be predicted that mutagenic chemical carcinogens would be more effective (potent) than nonmutagenic ones. Thus the latent period for cancer production would be expected to be shorter.

It is well known that in man cancer of the skin occurs mostly in exposed parts of the body (face and hands). On the assumption that the favorable terrain is formed initially by the random groupings of mutated cells, there should be little or no difference in the frequency of these regions of favorable terrain in different parts of the body surface. Therefore, contributing factors must play an important part in the induction of cancer of the skin in exposed areas. Cancer of the skin of the face is particularly prevalent in farmers (especially in Australia). In all cases of overexposure to sunlight in a dry climate, the skin shows general damage and local inadequacies to maintain its integrity. It is in such areas that cancer usually develops. In an area in which a normal skin surface cannot be maintained because the epithelial cells are not functioning properly, there is a constant effort to replace the superficial cells, and hyperplasia results. This is of interest in connection with the APA hypothesis in that cell proliferation indicates that the concentration is low, and a cancer cell in this region can divide and establish a tumor.

Since ultraviolet radiation (UV) is known to be mutagenic (when it can penetrate to the nucleus of the cell), this component of solar radiation may be the chief causative agent. In this case the mechanism of cancer induction is essentially the same as for chronic exposure to ionizing radiation. It must be borne in mind, however, that the penetration of UV into the skin is very superficial. This may account for the lower incidence of cancer of the skin in brunettes than in blondes and for the much lower incidence in the nonwhite population of the United States. In 1947 the age-adjusted incidence of cancer of the skin of the face, head, and neck was 39 per 100,000 in whites and 2.5 per 100,000 in nonwhites.[8] For primary cancers in other sites the difference between whites and nonwhites is very much smaller and may be higher or lower for whites, depending on the site and type of cancer. In this connection, it is most significant that the incidence of cancer of the skin of the lower extremities (which are usually covered by clothing) in 1947 was 1.4 and 1.1 per 100,000 in whites and nonwhites, respectively. The corresponding figures for the upper extremities are 3 and 0.3.

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* Obviously, random mutations in the descendants of these cells are of no importanc because they are soon replaced by new cells. Note, however, that some descendants may be mutated into cancer cells (for example, squamous cell carcinoma).

Chemical carcinogens constitute the next logical class of contributory factors. Some of these are known to be mutagenic and, therefore, the mechanism of cancer induction may be assumed to be essentially the same as that of ionizing radiation or UV. Those that are not mutagenic must be assumed to provide the proper terrain for the division and multiplication of a cancer cell that happens to be in the skin area to which the carcinogen has been applied, after it has been produced by the spontaneous mutation of a normal cell. It should be noted in this connection that a precancerous state, with hyperplasia, is generally present in such cases. The mechanism proposed here is indeed applicable to numerous cancerogenic agents of all kinds, including mechanical irritation of sufficient degree and duration.

Hereditary factors promoting the development of skin cancers may be considered next. While the possibility cannot be excluded that in some individuals the genetic constitution of the cells is such as will facilitate the spontaneous mutation into cancer cells (which would be inheritable) the writer thinks that, if this factor exists at all, it plays a very minor part. The reason is that according to the postulated mechanism, spontaneous somatic mutations provide an abundance of cancer cells (see below) and that the development of a tumor depends on the juxtaposition in space and time of such a cell and a favorable terrain. Therefore, one should look for hereditary factors that promote the formation of a suitable terrain. Dry, scaly skin provides such a factor. This correlates with the higher incidence of skin cancer in older people whose skin has become drier, less pliable, and scaly in spots. Therefore, the effect of inherited dry skin is essentially to lower the chronological age at which cancer may develop. This is supported by observations on radiologists whose hands have been chronically exposed to radiation. Another example of an hereditary contributory factor is the presence of pigmented nevi. They may be present at birth or may appear later. They are quite prevalent in white people, but malignant melanoma, which arises from them, is rare. This again emphasizes the dual mechanism in cancerogenesis. In general, a nevus is quite small in volume, and the chance that one of its cells will be spontaneously mutated into a cancer cell must be extremely small. Also, the terrain must be favorable at the proper time.

The presentation so far has been in terms of cancer of the skin, because the gradual changes that take place with age and those that lead to neoplasia are readily observable. Analogous spontaneous changes must take place in other body tissues modified, however, by differences in morphology, structure, function, and innumerable other characteristics. An important difference between skin and any other organ is due to the fact that the skin, being on the exterior of the body, is more exposed to all sorts of deleterious agents and mechanical damage. This accounts, at least in part, for the high incidence of skin cancer, which, however, does not contribute much to cancer mortality, because (with some notable exceptions) skin cancer is readil detected and eradicated before it has metastasized.

In considering tissue changes in other organs, it should be borne in min that, according to the mechanism postulated here, a local disturbance (o the right kind) in a small volume of tissue is sufficient to provide a favorabl terrain for the development of a tumor. Such local disturbances may occu with age because of the accidental grouping of randomly mutated cells because of local trauma, because of hormonal changes, because of inherite or neonatal local peculiarities in tissue structure that appear in later life o become important as other tissue changes caused by the aging process tak place, and, no doubt, for many other reasons. These small local disturbance in other organs are not readily observable for obvious reasons. Cowdry, who has made an extensive study of the changes that occur in the epidermi with increasing age, states: "As we grow older our epidermal cells are subjected to strains and stresses of many sorts. The orderliness in their live is to some extent broken up. Their mitotic rhythm may be upset. They seem to waver between extremes of hyperplasia and atrophy, mineralization and demineralization, pigmentation and depigmentation, and they suffe from inadequacy of epidermal appendages (hairs and glands). These ar changes not diffuse and uniform, but in most cases fairly sharply localized i foci especially in exposed areas of epidermis. In attempting to survive by adaptation to such conditions it is not surprising that malignant transformations are quite frequent."

In connection with the possible occurrence of similar local disturbance i other tissues, Cowdry says: "One wonders whether examination of the epithelial linings of the respiratory, digestive and urogenital systems in simila whole mounts, if that were feasible, would also bring to light many localize areas showing age changes and which might even fail to receive attention i the study of serial sections. However, they would hardly be so pronounce as in the epidermis, for the epithelia in question occupy more protecte locations. It is unnecessary further to describe cellular populations givin rise occasionally to epithelial cancers (carcinomas)."

It will be noted that the existence of local "imperfections" in skin, visibl by the naked eye or through the microscope, is well established. It is wel known also that they increase in number and undergo changes with age. A least some of them constitute the foci in which cancer eventually develops The existence of a similar process in other tissues may be inferred. These local imperfections may arise in a variety of ways: they may be inherited just as the shape of the nose may be inherited; they may result from somati mutations occurring in the embryo (mosaics) or from a chance grouping o mutated cells occurring later; or they may be due to the action of physical chemical, or biological agents, or to other causes. One may think of thes local imperfections in tissues as being analogous to the imperfections in th orderly arrangement of atoms in a crystal. It is very difficult to "grow" perfect crystal. It is reasonable to suppose that during organogenesis— very critical process—local tissue imperfections could readily arise for variety of reasons. Some of these imperfections may be corrected later a indicated, for instance, by the disappearance of some birth marks in babies, or the gradual improvement in the appearance of surgical scars. Incidentally, the presence of local tissue imperfections produced in embryonic life may well account for the high incidence of some types of cancer in early life.

It is now necessary to make some quantitative estimates to see whether the dual process for the induction of cancer postulated here can account for the observed incidence of cancer of different types and its variation with age. We shall assume that the somatic mutation rate calculated from the age-specific mortality rate curve for man applies to the mutation of a normal cell into a cancer cell. This is 7.2×10^{-5} per gene per generation, or 2.4×10^{-6} per gene per year. It is reasonable to assume that, if the production of the postulated APA is controlled by a specific gene in the haploid cell, the two corresponding genes of an allelic pair in the diploid cell must be mutated (in a certain way) in order to stop the production of APA and thus, according to our mechanism, produce a cancer cell. Accordingly, if these cells are not eliminated in some way or another, the number will increase as the square of the age, assuming a constant number of cells in the body to begin with. The coefficient will be $(2.4 \times 10^{-6})^2 = 5.8 \times 10^{-12}$ and the number of cells that have been mutated to cancer cells at time t will be $N = N_o \times 5.8 \times 10^{-12} \times t^2$. To simplify the discussion, we may let N_o represent the total number of cells in the average adult body, and we may reckon time, t, from adulthood on to old age. Assuming that the average body cell is somewhat less than 10 μ in diameter, $N_o = 10^{14}$ approximately. Then, if each body cell is just as likely to be mutated into a cancer cell, $N = 10^{14} \times 5.8 \times 10^{-12} \times t^2$, and the number of cancer cells in the first year of the assumed time scale is 580. The number is obviously too large, and we need not pursue this further.

If it is assumed that the cancer cell must find itself in a favorable terrain before a tumor can develop, we can arrive at a figure approximating the observed incidence of cancer in man by choosing an appropriately small total volume for the tissue regions in which the terrain is propitious for the growth of a tumor. Plotting cancer incidence for any particular site against age on log-log paper, we find that the incidence increases as the nth power of age in a representative region of the life span. For most sites the value of n is about 4 or 5. From the assumption that the somatic cell must be mutated in two specific loci to produce a cancer cell, we get $n = 2$. Therefore, the difference must be accounted for on other grounds. Obviously, the difference may be made up by making a variety of assumptions, but this is not a fruitful procedure at this stage. For our present purpose it is sufficient to point out that the two-loci mutation hypothesis leads to an overabundance of cancer cells, which is a necessary requirement if the development of a tumor is to be attributed to the juxtaposition of a cancer cell and a favorable terrain. On this basis the increase in the incidence of cancer with a power of the age greater than two indicates that the foci of favorable terrain also increase rapidly with age, which is qualitatively supported by observations—at least in the case of the skin. As to the mechanisms by which these foci are formed and increase in number with age, many possibilities exist, but it seems that they must remain just within the realm of possibility for some time to come.

I feel that somatic mutations probably play a part in the development of foci of favorable terrain, as part of a general aging process based on somatic mutations—the latter explanation being plausible, but as yet unproved. The great difference in the incidence of skin cancer in exposed parts of the body between whites and nonwhites may be ascribed to the effect of sunlight, probably the UV component. If this is so, it supports the somatic mutation mechanism and, at the same time, it indicates that the spontaneous mutation rate (not including the mutation rate attributable to exposure to sunlight) is responsible for a small fraction of the skin cancer incidence in whites, roughly equivalent to the skin cancer incidence in nonwhites. From this it may be surmised that the role of spontaneous somatic mutations in producing foci of favorable terrain in other organs may not be so prominent. It is more likely that local tissue imperfections occur for a variety of reasons, but somatic mutations may turn some of them into favorable terrain for the division and multiplication of a cancer cell produced therein.

I acknowledge that others have had similar ideas about the aging process and/or cancerogenesis and have been aware of this fact since I first presented an outline of my views in December 1956.[10] The similarity between my views and those of others is closest in the case of the theory proposed by Osgood.[11] I thank Patricia McClement for fitting a four-hit curve to the age-specific mortality data of FIGURE 1 and for general help in the preparation of this paper.

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QUANTITATIVE COLONIAL GROWTH OF SINGLE MAMMALIAN CELLS

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The development of a simple methodology for growth of discrete, macrosopic colonies from cells of warm-blooded animals *in vitro* has made possible quantitative studies of cell reproduction, genetics, and interaction with viruses.[1, 2] The growth of mammalian cells in a particular medium can be expressed in terms of reproducible, numerical parameters of plating efficiency—that is, the number of cells in the population capable of initiating self-sustaining multiplication, duration of the lag period before growth is initiated, and the generation time in the exponential reproductive phase.[2] The use of X-irradiated feeder cells has demonstrated that single cells possess molecular requirements for growth different from those of large, closely packed cell populations, and has made possible studies of specific cell-cell interactions.[3, 4] Studies of the macromolecular nutritional requirements of pure cell lines have revealed the need for at least two protein constituents, albumin and a high molecular-weight gamma-globulin (electrophoretic mobility of -4.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8.5) that appears to be a constituent of the fetuin system.

The use of single-cell plating has made it much simpler to establish clonal cell lines of mammalian cells. Such strains have been isolated and maintained in luxurious growth for years, during which genetic characters such as nutritional requirements, chromosome count, and morphologic behavior have been stable. Even cell lines derived from originally malignant tissue such as the HeLa carcinoma of the cervix have, after 2 years of cultivation, been maintained with a uniformity indicated by the fact that more than 80 per cent of the cells of clonal cultures have the same chromosome count. Stable, differentiating, genetic characters, found either to occur naturally in cell cultures or to be produced by means of ionizing radiation, include characteristic nutritional requirements for single cell growth, chromosome number and morphology, virus resistance, and the degree to which the cells tend to elongate on a glass surface. The concepts and techniques of microbial genetics appear to be directly applicable to the mammalian cell.

Studies of the effect of ionizing radiation have made possible accurate measurement of the mean lethal dose to the reproductive apparatus of the individual cell. This value has been demonstrated to be approximately 100 r, and to vary by no more than 50 per cent for cells taken from young or old individuals, cultivated for long or short periods *in vitro*, exhibiting euploid or hyperploid chromosome conditions, originating in a variety of either normal or malignant human tissues, or demonstrating either epitheloid or fibroblastlike morphology. These data, together with other considerations, have demonstrated that the primary radiobiological damage in the mammalian cell is lodged in the genetic apparatus, and most probabl consists in aberration at the chromosomal level. This conclusion was confirmed by the demonstration of the production of large numbers of mutations many of which display modified chromosomal constitution, by doses o radiation equivalent to 5 to 10 mean lethal hits. These considerations hav been applied to the explanation of several aspects of the mammalian radiatio syndrome.[5, 6]

Application of these techniques to the study of interaction betwee mammalian cells and viruses has made possible determination of the poin in virus infection at which single cells lose the ability to reproduce.[7] Thi permits quantitative titration of viruses on the basis of this cell lethal activit and provides means of following processes of virus penetration and interference. Virus-resistant cell mutants have been obtained and grown int new, stable, clonal stocks. Some of these have proved capable of carryin an otherwise lethal virus in a stable association with the cell, so that individual cells can display a double potentiality: under certain conditions, eac cell multiplies to form a normal colony; under other circumstances, however each cell liberates lethal virus and is itself destroyed. This cellular virus-carrier state may be very important to an understanding of latent virus infections in mammals.[8]

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CELLULAR CHANGES INDUCED BY RADIATION*

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At a recent Ciba Foundation symposium, van Bekkum[1] stated: "Biochemical changes that can be demonstrated before structural damage to the cells becomes apparent are of the greatest interest, because these may be expected to be closely related to or even identical with the primary biochemical lesion." For more than a decade our tissue culture laboratory has been guided by such a point of view. To achieve our purposes, an eclectic position has been assumed in the use of a wide variety of methods for cell cultivation, but with special attention given to phase-contrast microscopy and the development of cinematographic devices, including instrumentation for the simultaneous recording of the time, temperature, and action potentials. More than 250,000 feet of 16-mm. film records have been accumulated that serve as a reference library regarding the behavior of various species of cells *in vitro.* We have been especially concerned with activities such as membrane movement, pinocytosis, the mobility of mitochondria, the motion of aggregated bodies in the juxtanuclear zone, nuclear rotation, contractions of the nuclear membrane, and the rhythmic pulsation of whole cells or their processes. These records have invited quantitative analyses in relation to metabolic activity. While an immense amount of work remains unfinished, preliminary inroads have been made in the description of some activity manifestations of the cell with a group of techniques designed to convert dynamic cinematographic records into graphical summaries. The analysis of nuclear rotation in the epithelium of adult human nasal mucosa (Pomerat[2]) and the ascent of vacuoles in the axoplasm from the growth cones of regenerating neurons from chick dorsal root ganglia as a result of pinocytosis (Nakai[3]) are representative of this effort. Cinematographic records have been particularly useful in the study of events taking place in the course of mitosis. Occasionally, film sequences accumulated with other goals in view have made invaluable data available for the study of cell division (Hsu,[4] Hsu and Moorhead,[5] Moorhead and Hsu[6]).

In attempting to apply *in vitro* findings regarding biochemical mechanisms involved in ionizing radiations to the injury observed in irradiated cells, it is hoped that the analysis of time lapse, phase cinematographic records may prove useful.

The review of the literature on the use of tissue cultures in the study of ionizing radiations by Stroud and Brues[7] is a valuable key to the contributions in this field. A summary statement regarding our own efforts was presented at the Conference on Research in Radiology held at Highland Park, Ill., on

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Materials and Methods

Culture procedures generally involved the use of the multipurpose chamber designed by Rose[8] since this offers parallel-sided glass surfaces with good optical qualities having a diameter of 25 mm. The main disadvantage of the system is that it requires a microscope working distance of at least 6 mm., but this is compensated by the fact that, with its very strong framework and thick pure gum, rubber gasket, the chamber absorbs shock during shipment. These properties have favored its use in preliminary studies on the effect of cosmic radiations on cells in high-altitude balloon ascensions.

Cell cultures generally consisted of "strain" lines, such as the HeLa clonal S3 (Puck), which we have employed extensively for radiation and other studies with the view of accumulating a large body of data on a particular cell species. However, ten other human strains and about fifteen species of cells from primary explants have been used. Details of the materials employed will be described in the sections dealing with the results obtained.

Photographic records were made of living preparations with AO and B & L (long working distance) phase objectives, both on still and cinematographic units.* Dual setups permitting the simultaneous recording of irradiated and control cultures have been found particularly useful (Pomerat and Smith[9]). Of special interest is the technique using a double prism system (B & L comparison eyepiece) which permits the recording of cellular events in sister cultures variously exposed to radiation from two microscopes on the same 16-mm. film strip.

Stained preparations were usually made after fixing cultures 6 or 12 days after the period of irradiation. The Giemsa-May-Grünwald combination as suggested by Werner Jacobson was found most practical. Many specimens received prefixation treatment with a hypotonic saline solution to achieve chromosome spreading. The squash method recently advocated by T. C. Hsu (personal communication) involves treating cultured cells with colchicine, suspending them in a hypotonic solution, fixing with acetic alcohol, and staining with acetic orcein.

Radiation methods. Cultures were transported to the Radiobiological Laboratory of The University of Texas and the United States Air Force in Austin, Texas, for irradiation in Thermos bottles and in an insulated box to maintain temperatures at approximately 37° C.

For gamma radiations, cultures were exposed to a high-level cobalt-60 facility designed to give a uniform flux over an area 2 in. × 6 in. For total doses less than 1000 r, the rate was 200 r/min., while values of more than 2000 r were delivered at 2000 r/min.

In a limited number of experiments using X radiation, the source was a self-rectifying, half-wave, 260 kvp, 18 mAmp. therapeutic unit with an inherent filtration of 0.25 mm. Cu plus 2 mm. Al. Voltages used were 50 kvp and 250 kvp. For the 250 kvp exposures, radiation factors were current, 18 mAmp.; filtration, 1 mm. Al and 0.25 mm. Cu; dose rate to the field of irradiation, 500 r/min. For the 50 kvp irradiation, the radiation factors were current, 18 mAmp.; filtration, 0.032 mm. Al; dose delivered to the field of irradiation, 23 r/min.

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* Rolab, Sandy Hook, Conn., and Electro-Mechanical Development Company, Houston, Texas.

Results

Minimal radiation dose for irreversible injury to cells in culture. In a series of pilot experiments, Walther Hild of this laboratory was able to reconfirm reports in the literature of tissue culture that very high doses are needed to achieve irreversible damage to monolayers of cells *in vitro.* Approximately 140,000 r of gamma from a cobalt-60 source was required to destroy cells in Rose chambers within a 24-hour period of incubation. This value was obtained for the Henle strain of intestinal epithelium, and appeared to be of the same order of magnitude for several species of cells from primary explants. The main importance of this phase of the work was the demonstration that with 80,000 r or more the phenol red indicator used as a guide to judge over-all cell activity was decomposed. All subsequent experiments were performed on cells from which all fluid nutrient was removed during the period of irradiation. In turn, this modification in the procedure led to the observation of the lowering of the lethal dose. In contrast to a value of 140,000 r for fluid-filled chambers, irreversible injury to the Henle strain of intestinal epithelium dropped to approximately 80,000 r.

This result suggested a series of experiments on the effect of the gaseous environment on sensitivity of cells to radiation. It will be appreciated that, since fluid removal from the Rose chamber is readily effected by inserting two No. 25 gauge needles through the rubber gasket that forms the wall of the chamber, displacement of the nutrient with various gases is easily achieved. A report in preparation is concerned with injury to cells irradiated in the presence of a mixture of 96 per cent N_2 plus 4 per cent CO_2, air, and a combination of 95 per cent O_2 and 5 per cent CO_2. Results are in keeping with observations obtained with other living systems in which radiosensitivity has been found to increase sharply with a rise in the level of O_2, but with little additional effect at concentrations above 20 per cent. The tissue culture method appears to offer challenging opportunities in this area with special reference to alterations in cell organoids, particularly in the study of mitochondria in relation to the factor of oxygen levels during irradiation.

Giant cell formation in various culture systems. The report of giant cell formation following exposure of cell cultures to both X and gamma radiation by Edith Paterson[10] in 1942 has been greatly enriched by the contributions of Puck and his associates. Radiation-induced giant cells are characterized by bizarre multilobed nucleoli and nuclear membranes that show surface wrinklings and marked indentations (FIGURE 1). While the phenomenon also has been observed in unirradiated cells, such nuclei often exhibit surface contractions and twitching movements in addition to complete rotation. Since the immediate cytoplasmic environment of such nuclei is rich in actively mobile, long, filamentous mitochondria, one is tempted to speculate

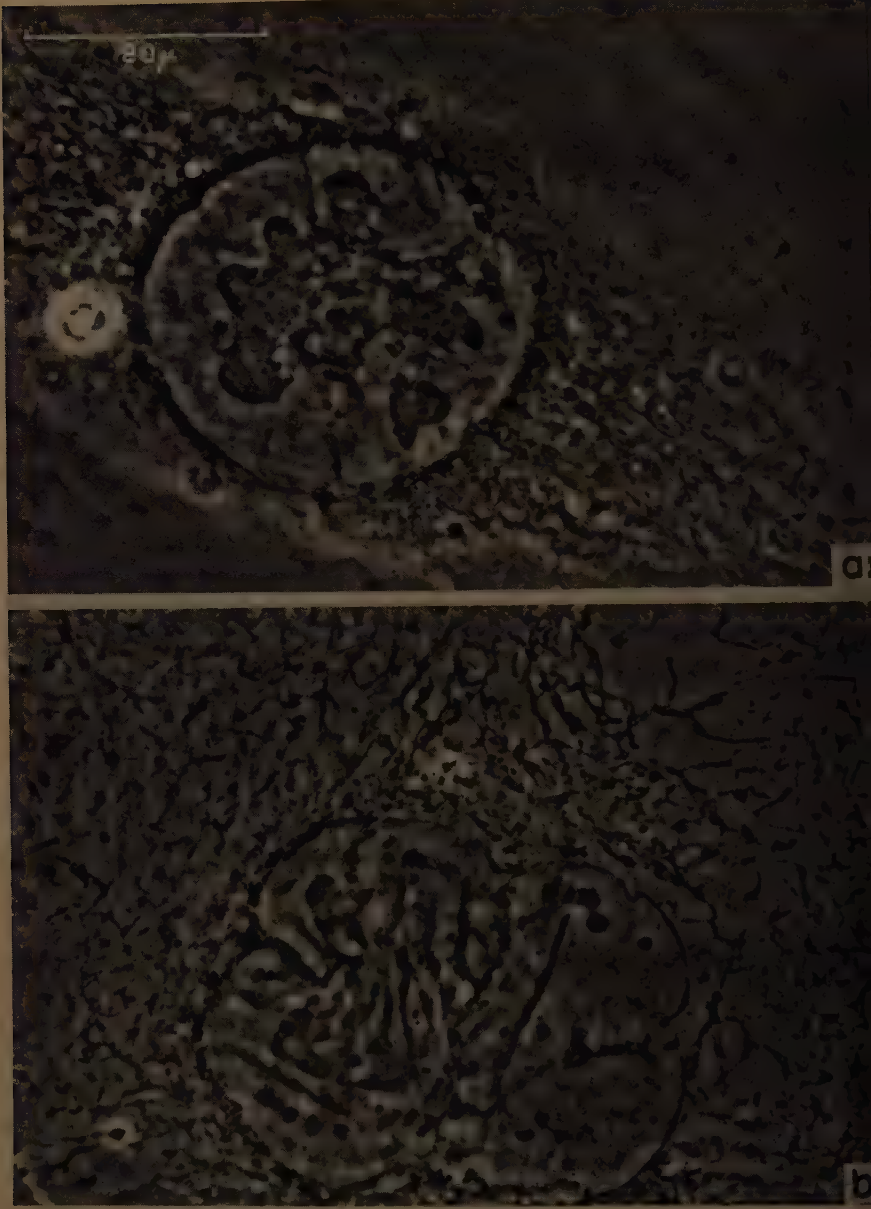

FIGURE 1. Giant cells in cultures of the human fetal intestinal cell strain of Henl
The cultures received 2000 r of gamma irradiation from a cobalt-60 source on the thir
day after transfer and were photographed with a dark phase-contrast optical system 25 day
later. Note the enlarged and bizarrely shaped nucleoli, wrinkled nuclear membranes, an
the rich perinuclear aggregation of filamentous mitochondria. The magnification for bot
photomicrographs is given by the scale shown in 1a.

that these morphologic and activity manifestations may be related to metabolic events that make such cells valuable in "feeder" layers, as described by Puck and his associates.

In the quest for end points that could easily be quantitated, Y. Nakanishi in our group found no significant difference in the ratio of mitotic phases between cells of the HeLa S3 clone that received 100 r of gamma irradiation from a cobalt-60 source and controls fixed 12 days after irradiation. However, under these conditions 51 of 2042 control cells (2.49 per cent) had nuclear buds, in contrast to 110 out of 2040 irradiated elements (5.37 per cent). At 750 and 1000 r of gamma irradiation, the number of nuclear buds was not significantly different from those of corresponding controls. At these dosage levels, cultures fixed on the twelfth day showed that the number of multinucleated cells was increased. There were 8 of 2001 control cells with more than 3 nuclei (0.4 per cent), as compared with 14 of 1028 cells (1.36 per cent) in the 750 r series. The controls for the 1000 r dose had 7 multinucleates of 2113 (0.33 per cent) against 67 of 1114 (6.0 per cent) cells in the treated cultures. Nuclear lobulation, single and multiple buds, and nuclear masses that appear to vanish in the cytoplasm in cultures irradiated both with gamma and neutrons are illustrated in FIGURE 2.

We have been concerned with the formation of giant cells in relation to (1) so-called cell strains, (2) outwandering cells from primary explants, and (3) elements having survived several culture passages *in vitro* without having become established as "strain" lines.

Cell strains. In a report (Pomerat *et al.*[11]) which includes a brief review of the work by Puck and Marcus,[12] we have presented illustrations of giant cell formation within 12 days following irradiation with 2000 r and 4000 r from a cobalt-60 source from 3 strains. These lines were derived from malignant tissue (HeLa, clonal S3—Puck; adenocarcinoma of the lung—Maben; epidermoid carcinoma of the gingiva—KB of Eagle), and from 6 strains that were developed from presumably nonmalignant sources (fetal intestinal epithelium—Henle; the conjunctival and hepatic epithelia lines of Chang; adult human skin NCTC, clone 2414; and two lines from our laboratory; synovial fluid—McC and a fetal fibroblast). Attention has been drawn to the fact that the modal chromosome numbers that have been found for these cell strains range from 67 to 83.

Cells from primary explants. In contrast to results obtained with cell strains, outwandering elements from liver explants from a human fetus, from a puppy, from a young monkey, from monkey lung, from adult human meninges, and from the components of granulating tissue never were found, by a technique identical with that employed for its demonstration with cell strains, to produce giant cells.[11] It was assumed that the chromosome numbers of the majority of the cells in this series were grouped around their diploid values.

Cells from subcultures not established as strains. In rechecking Paterson's original article,[10] it was found that she had obtained giant cells from second-passage cultures of chick embryo choroid-scleral tissue that was also subcultured after irradiation. Since the radiation dosage levels that she had

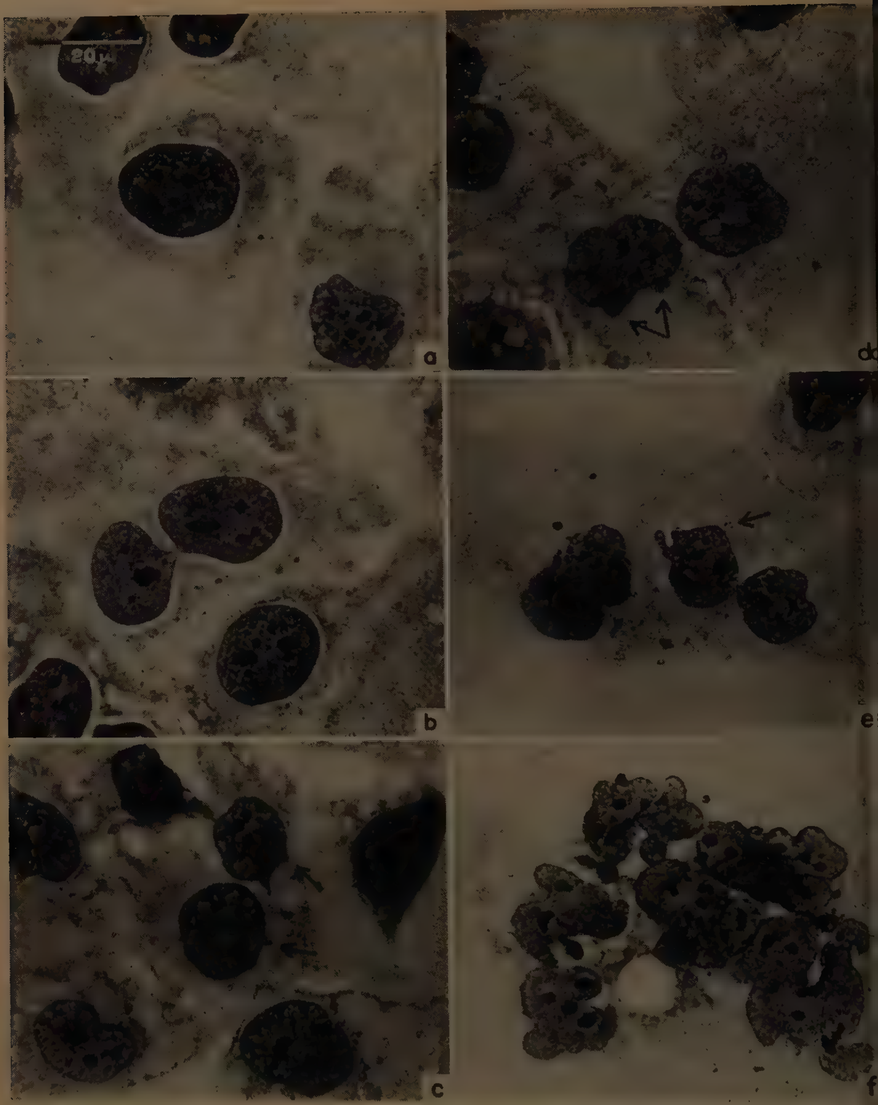

FIGURE 2. Nuclei of HeLa clone S3 cells that received 100 rep of neutron irradiatio (polonium source) 1 day after transfer to Rose chambers and fixed 12 days later wit absolute methyl alcohol and stained with Giemsa-May-Grünwald. The scale for al photographs is indicated in 2a, the central element of which shows a nonlobed nucleu with multiple nucleoli but without buds; (2b) a bilobed nucleus; (2c) two nuclei with singl buds (arrows); (2d) a nucleus with two young outpushings (arrows) and a nucleus fro which a bud has become almost completely separated; (2e) the arrow indicates the locatio of a nuclear bud which is believed to be merging into the cytoplasm; and (2f) multip multilobed nuclei in a single cell. This figure was kindly made available for this repo by Y. Nakanishi.

employed were of the same order as those that we had been using, cell outgrowth from chick embryonic choroid-scleral explants was irradiated according to the experimental conditions described in our work on giant cell production. Primary explants did not produce giant cells, but second-passage cultures yielded large, fat-laden elements closely resembling those in the illustrations published by Paterson in 1942 (Harper *et al.*[13]).

In a recent study (Hsu *et al.*[14]), chromosome counts made on adult human synovial cells at the time of each culture passage revealed that, while the diploid value was dominant at the outset, the total number of chromosomes in the 40-to-50 group fell with time. In the eighth subculture no chromosomes were found in the diploid range, but counts extended above 55 to more than 200. By the ninth passage, the most frequent values appeared to have become grouped in the range of 100 to 135. Current work in this laboratory by Y. H. Nakanishi has shown that chromosome numbers for cells in the initial outgrowth of both human fetal and kitten lung are located predominantly at the diploid mean. After subculture, the degree of aneuploidy progressively spreads in both directions, so that within 3 or 4 passages little evidence of a peak remains in the histogram at the level of the accepted somatic number. As a working hypothesis, therefore, it is believed that the increased aneuploidy resulting from the adaptation of cells to *in vitro* life may be related to the development of giant cells following irradiation of passage cultures. In keeping with this view, the characteristic rapid, massive production of giant cells following the irradiation of strain cultures is probably correlated with the heteroploidy regularly encountered in these cell lines.

Cineanalysis of the development of giant forms in living cells following irradiation. More than 6000 feet of 16-mm. phase contrast, time-lapse cinematographic records, generally made from 1 to 5 days following gamma irradiation with 2000 r and 4000 r from a cobalt-60 source, are available for analysis. The cells employed include the HeLa S3 clone, intestinal epithelium (Henle) and adult human skin (NCTC clone 2414). One of the advantages resulting from the use of these cell species was their relatively high resistance to the injurious effect of illumination used for photography. Cine records made of a particular field of cells with an 8-mm. objective, but without an ocular, at one frame per minute for as long as 5 days did not show evidences of damage beyond what could be found in the same chamber outside of the path of the light beam, or in corresponding cultures that were not employed for photomicrography. Thus, it was possible to follow the natural history of the morphology and activities of particular cells for a crucial period following exposure to radiation. Selected film frames abstracted from the records were enlarged and used to illustrate the most significant events observed in the course of transformations leading to the production of cells of giant size (Pomerat *et al.*[15]).

Postirradiation, gradual cell enlargement is most commonly encountered for all the cell strains that have been studied. Such progressive increases in the mass of protoplasm are not accompanied by evidences of nuclear division.

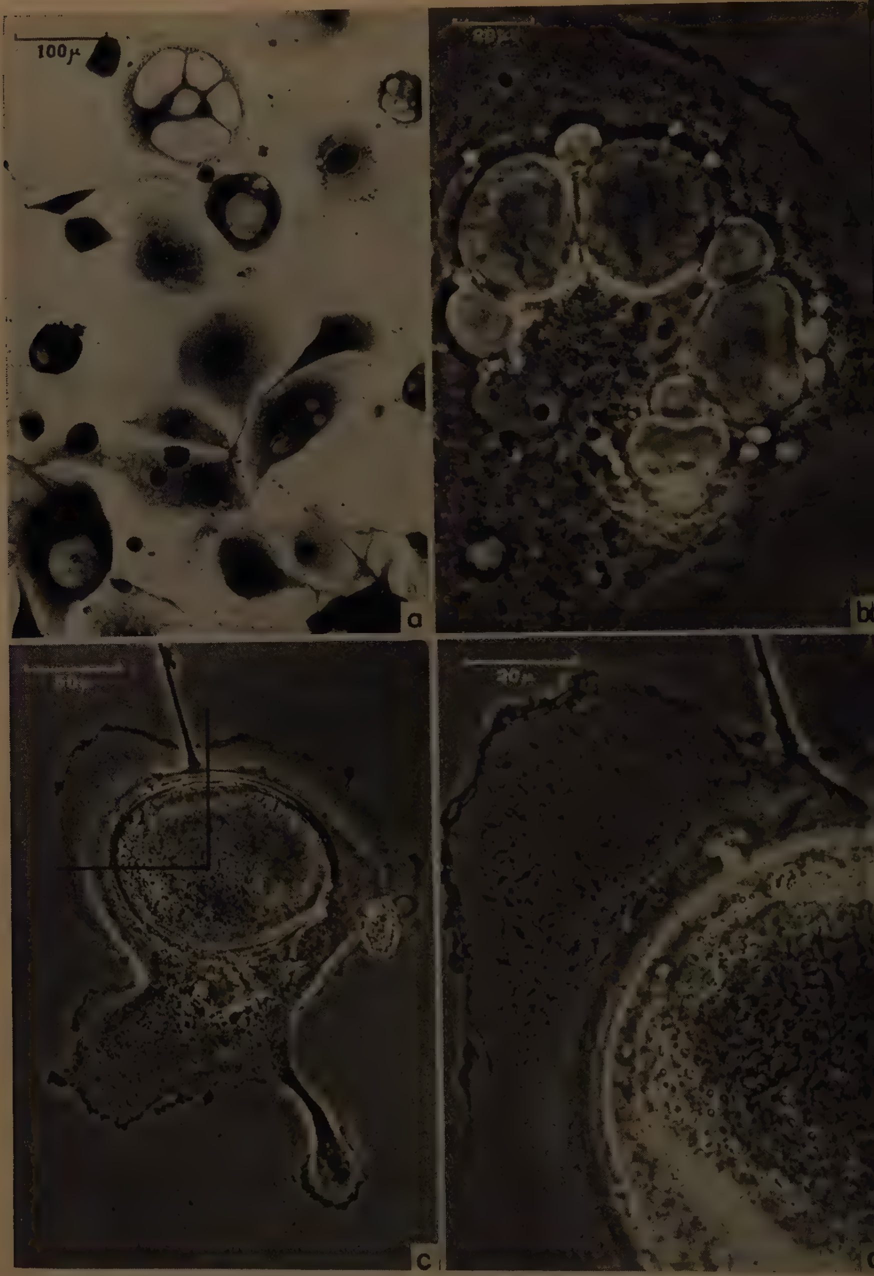

FIGURE 3. Adult human skin clonal strain (NCTC 2414) cells that received gamm irradiation from a cobalt-60 source. (3a) Elements in a culture that received 2000 4 days after transfer, fixed with methyl alcohol and stained with Giemsa-May-Grünwal 12 days later. Note the presence and variety of intracellular vacuoles in the majorit

Teloreduplication, which has been defined as the simple fusion of daughter cells during or after cytokinesis, is relatively frequent. Generally, it results n a cell with at least two distinct nuclei.

Cells with single as well as with double nuclei have been followed and proved to be successful in establishing multiple micronuclei without cytokinesis. The records include a cell with 6 nuclei in a culture of the skin strain that produced some 60 micronuclei.

The viability of cells showing abnormal divisions as proved with cinematographic technique requires much further study. Repeatedly, it has been proved that elements with multiple nuclei can enter a second division to produce even larger numbers of micronuclei. Observations on cell lineage with special reference to the imposition of experimentally controllable njuries are greatly to be desired in the development of genetic concepts for the origin of cancer.

Radiation cytopathology recorded in living cells. In the course of observations on irradiated cells in culture, a variety of intranuclear and cytoplasmic anomalies have been observed. While multiple small vacuoles frequently have been seen in the nuclei of cells from the Henle strain of intestinal epithelium, the most regularly reproducible lesion has been recorded in cells of a skin strain.

A study undertaken to define the characteristics of long-term, large-scale cultures of human epidermis as a cooperative project between the Tissue Culture Section at the National Cancer Institute, Public Health Service, Bethesda, Md., and the Tissue Bank of the Naval Medical School, Washington, D. C., led to the establishment of a strain of epithelium, to be known as NCTC 1769, from a 52-year-old* male who had died of coronary occlusion (Perry *et al.*[16]). A clone strain begun in November 1953 was kindly supplied for our studies by Wilton R. Earle with the designation NCTC-clone 2414-12-4.

Following irradiation both with 2000 r and 4000 r from a cobalt-60 source, vacuoles regularly have been seen to develop in the cytoplasm of at least 20 per cent of the giant cells within approximately 5 days (FIGURE 3). Vacuolar areas may be single or multiple, they increase in volume with time, and there is cinematographic evidence that their content may be expelled rapidly. Occasionally, the entire cell appears to consist of a globose mass anchored to the coverglass by means of an actively mobile peripheral membrane (FIGURES 3c and 3d). Fortunately, such cells are markedly resistant

* Corrected age—personal communication, V. P. Perry.

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of the enlarged cells. (3b) Characteristic appearance of multiple intracellular vacuoles n a living cell photographed with dark-phase optics 12 days after the culture received 2000 r three days after transfer to a Rose chamber. (3c and 3d) A cell that received 4000 r three days after transfer that was photographed with dark-phase optics 7 days later. The lesion consisted of a single large vacuole surrounded by a zone of cytoplasm with an active ectoplasmic membrane. FIGURE 3c represents the entire cell, while FIGURE 3d shows one quadrant at higher magnification revealing surface membrane wrinkles both over the vacuole and in the marginal cytoplasm. Each illustration carries its scale of magnification.

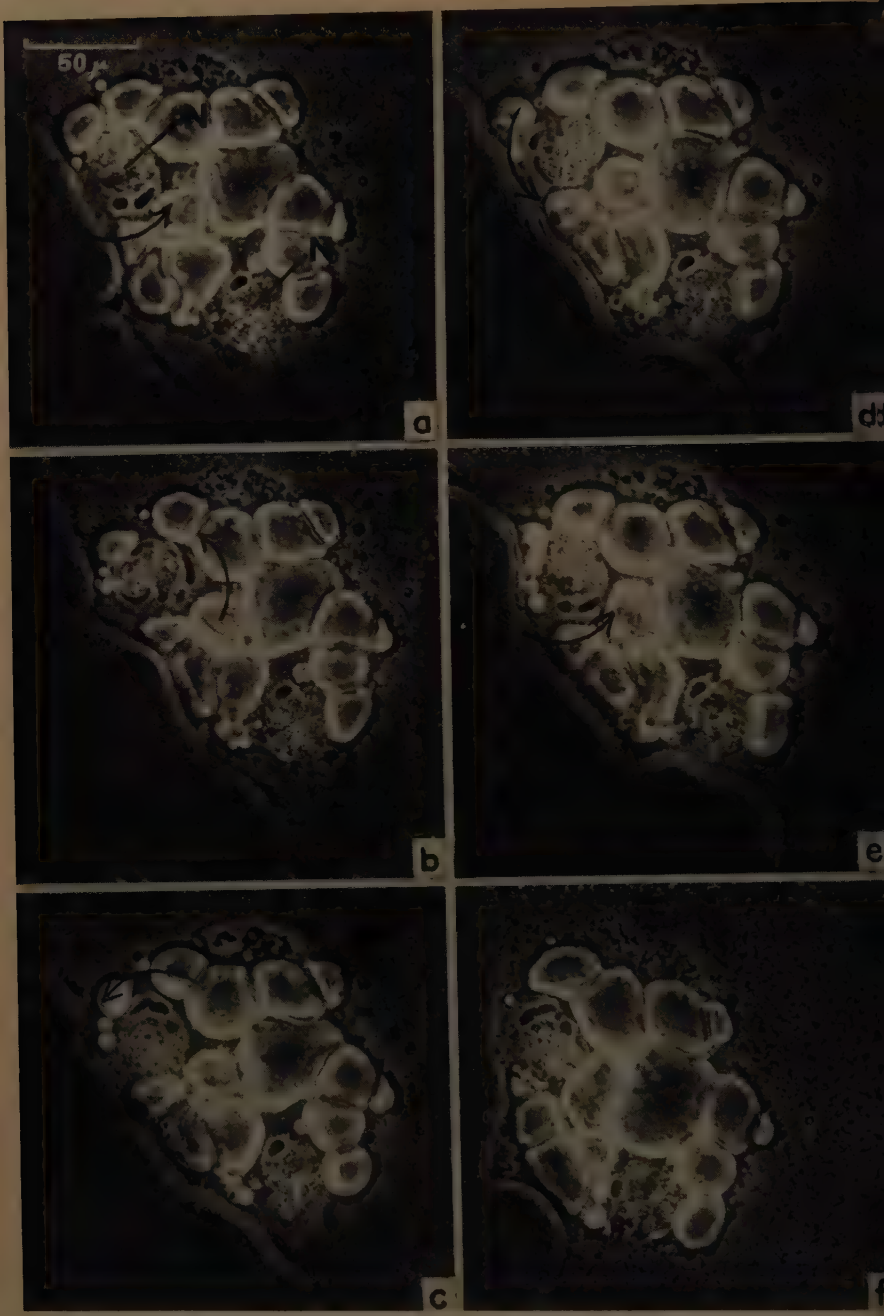

FIGURE 4. Selected 16-mm. film frames enlarged to show cytopathological injury to cell from the adult human skin clonal strain NCTC 2414 following with 4000 r of gamm

o the conditions essential for long-continued observations with cine tech- ique. For example, records include observations on the same field photo- raphed with an oil immersion objective at one frame per minute for as much s 7 days.

FIGURE 4 represents selected frames from a film record of the NCTC lone 2414 skin cell that had received 4000 r from a cobalt-60 source. The illustrations were made of activities observed during the eleventh day ostirradiation. Not only did this cell contain multiple cytoplasmic vacu- les, but lesions were seen in both nuclei. Highly refractile intranuclear articles showed rapid movement, and one of the nuclei performed a com- lete revolution within approximately three hours, as judged by the shifting osition of the optically dense nucleoli (see arrows shown in FIGURES 4a to 4d).

The NCTC clone 2414 cell has become morphologically very different rom outwandering epidermal elements obtained from freshly explanted adult uman skin, and its chromosome number is now elevated to a mean of 76 personal communication—T. C. Hsu). These changes probably are related o its capacity to develop benign growths in experimental animals. Further tudy is necessary to prove whether the lesions produced in the NCTC clone 414 cells as a result of irradiation are due to a viral agent or represent a pecial injury manifestation directly caused by ionizing radiations. Work in rogress by Leonard Hayflick in this relation includes the use of standard nethods for the demonstration of filtrable agents. Cells irradiated with 000 r (gamma) in bottles that showed characteristic cytopathology were apidly frozen (−70° C. in an alcohol bath) and thawed 6 times in the utrient medium. This material was spun in the No. 40 rotor of the Spinco ltracentrifuge for 2 hours at 35,000 rpm. After centrifugation, the content f each tube was arbitrarily divided into 3 segments and these were labeled ractions 1, 2, and 3. Fraction 3 represented the heaviest material. The fractions were held at −70° C. until used, when each was added to the utrient fluid of normal human amnion cell line cultures. Results obtained hus far are not definitive.

The cytopathology resulting from irradiation may provide a valuable tool, ot only for the study of cell transformations occurring in the course of ecoming established as cell lines, but also in the quest of so-called "masked" iruses in tumor tissues.

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radiation from a cobalt-60 source 3 days after transfer to a Rose chamber. The record as obtained with an 8-mm. dark-phase objective and no ocular at a rate of one frame per inute. Photographs were made during the eleventh and twelfth days postirradiation, ut activities during only somewhat more than 6 hours are presented in this plate. The ell contained multiple cytoplasmic vacuoles and intranuclear lesions in each of its two uclei (N). In addition to the rapid oscillatory movement of the granules in the intra- uclear area, one nucleus revolved. The direction of this activity is indicated by the rrows in FIGURES 4a to 4e, covering a rotation of 360° in approximately 3 hours, as can eadily be established by following the position of the 2 dense nucleolar bodies in this ucleus. (4a) Zero time; (4b) 1 min., 18 sec.; (4c) 1 min., 48 sec.; (4d) 2 min., 53 sec.; 4e) 3 min., 16 sec.; and (4f) 6 min., 43 sec. The magnification for all photographs is iven on FIGURE 4a.

In a pilot series no peculiar lesions have been found in primary cell cultures from 3 human brain tumors 12 days following irradiation with 2000 r an 4000 r from a cobalt-60 source. The program of these investigations i designed to include longer periods of observation with the use of mass cultures of tumors maintained through several passages.

Acknowledgments

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My colleagues, Walther Hild, Leonard Hayflick, T. C. Hsu, and Yuh Nakanishi, permitted me to use unpublished data for this report. Mrs W. Hild and C. Raiborn Jr. carried out procedures for cell culture, whil C. G. Lefeber and E. E. Pitsinger were responsible for photographic records To all, grateful acknowledgment is due for their enthusiastic and arduou contributions to the enterprise.

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MAMMALIAN CYTOGENETICS AND THE CANCER PROBLEM*

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The successful preparation of preliminary maps of germinal chromosome in the mouse by Slizynski[1] and by Griffen[2] has indicated clearly that cytogenetic studies and experimentation on mammals are entirely feasible. I the mouse a formidable array of classic genetic problems awaits cytogeneti analysis and guidance and, in the field of cancer research, an even greate number of questions may be answered at least partially through cytogeneti approaches.

The classic problems may require only the study of germinal cells, suc as the pachytene stage of gametogenesis, in which the phenomenon of synapsis provides twenty relatively large chromosomal tetrads representing th entire genome of the individual. Cancer problems, on the other hand require the difficult study of somatic cells in which synapsis is rare, variou degrees of polyploidy are common, and frequent instances of aneuploid can be demonstrated; any or all of these features may discourage an investigator who attempts to study the genetic composition of nuclei in malignan cells.

When my own first map of the germinal chromosomes of the mouse wa being tested for general usability, attempts were made to apply the knowledg of chromomere sequences to the identification of favorable large somati prophase chromosomes in tumor cells. A teratoma of ovarian origin, studie extensively by Fekete and Ferrigno[3] and Fekete and Griffen,[4] was found t produce, in both nodular and ascitic growths, numerous embryonal cell characterized by large and especially prominent chromosomes. At prophas these chromosomes often reveal clear chromomeric organization, particularl after staining with Sudan black-B and with the use of phase-contrast microscopy (FIGURE 1). Homologous chromosomes were occasionally detecte even though they lay remote from each other in the crushed and flattene nuclei; however, all attempts to identify even the most favorably spread an stained elements with those of the pachytene map proved fruitless. Up t this point, phase contrast had not been employed in pachytene microscop since it had early been decided that only the maximum resolution obtaine in apochromatic microscopy would be used in chromosome mapping. No however, it seemed obvious that the two types of cells must be studied wit the same type of microscopy. Accordingly, a new pachytene map was prepared with the use of phase contrast; this map is not yet considered accura for the smaller chromosomes, which require extensive checking.

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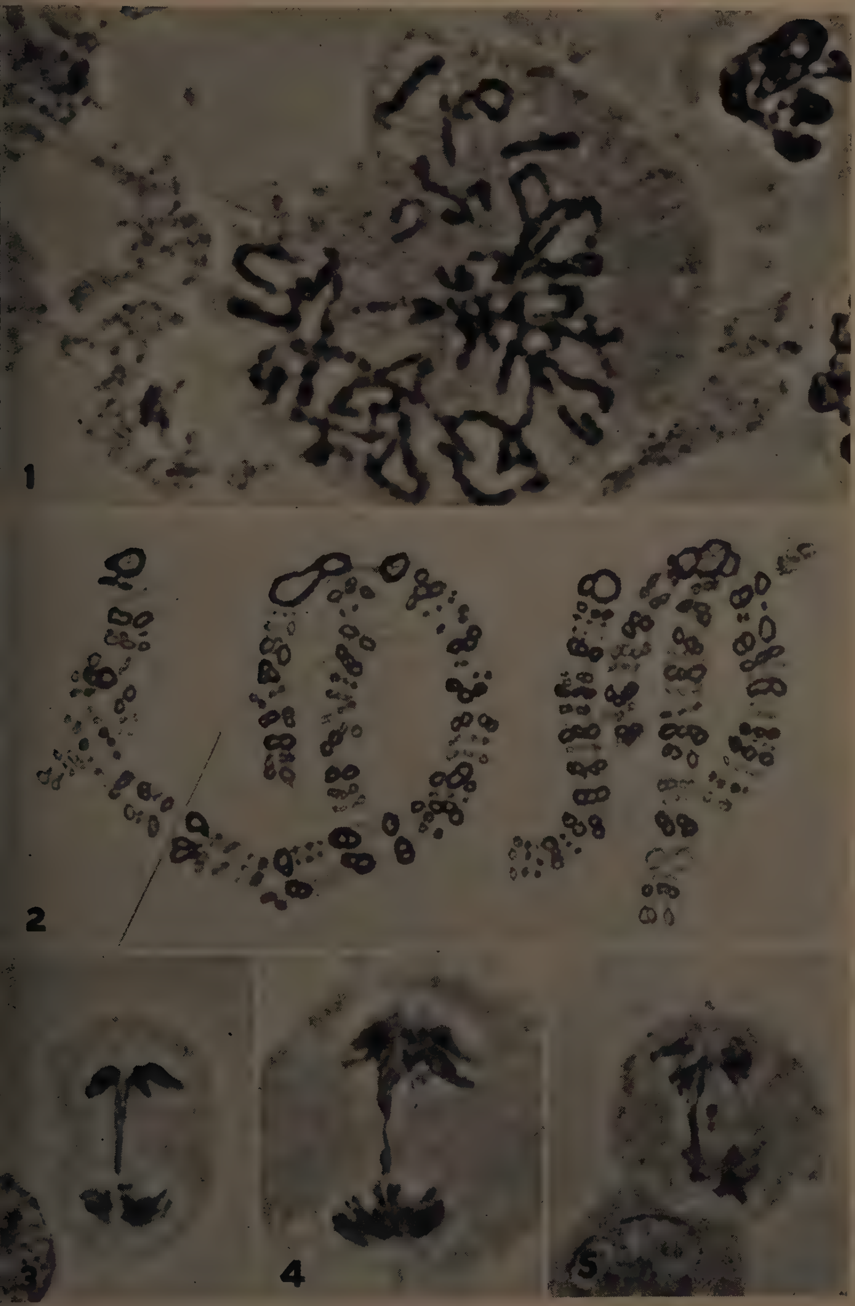


Figures 1 to 5

With the use of the phase-contrast map two of the larger chromosomes V and VIII, were readily identified in tumor prophases. Other chromosome seemed not to match those of the phase map, in either the number or th morphology of chromomeres; the map was found to be incomplete in tha the terminal portions of all chromosomes were not reliably represented. A clear and unmistakable termination point was needed for at least one end of each map element. The centromeres or spindle attachment points could satisfy this requirement, if they could be located, for all the centromeres o the mouse are terminal or immediately subterminal in position. In orde to identify these bodies, which neither Slizynski nor I had found in ou map constructions, I reviewed a study I had made in collaboration with Mary Warters[5] on centromere locations in the chromosomes of *Drosophil melanogaster*.

In *Drosophila* salivary gland cells, the spindle attachment regions of al the chromosomes are obscured in a loosely organized heterochromati "chromocenter." In this mass Warters and I had located prominen chromomeres that were clearly structures of the X and IV chromosomes each of which has a small and obscure second "arm" beyond the prominen first arms that bear all or most of the known X and IV genes. There ha been no apparent means of proving that these bodies were centromeres however, with knowledge of centromere morphology now required for th mouse work, the *Drosophila* problem was reopened, and a means for identifying the X centromeres was devised.

Through the irradiation of an X chromosome bearing a point known to b readily breakable by X rays, small ring chromosomes could be produced each consisting of (1) a fragment of the known genetic arm, broken at th "weak" point; (2) a portion of the obscure second arm with the weak poin attached to its stump; and (3) the centromere, proved to be present b successful passage of the ring through cell divisions. In effect, variou lengths of the second arm were to be deleted from the system, leaving onl limited areas of the original chromosome as possible centromere locations Accordingly, males from a stock bearing a translocation between chromosomes X and IV, with the dominant *Bar* eye shape at the breakage point were irradiated and appropriately mated. Among the progeny two sma rings of the necessary type were found, one having the greater part of th obscure second arm still present, and the other having a full deletion of th second arm, so that the *Bar* locus could be found attached directly adjacen to chromomere band 20 D of the known genetic arm.

With the removal of the obscure portion of the X chromosome, the chromocentral mass was largely clarified. Chromomere band 20 D was now foun to synapse consistently with the presumed centromeres of chromosome I and with similar structures in chromosome III, in each case forming a sma loose cluster of densely staining bodies. In all nuclei studied, each pair synapsed chromosomes is borne by a total of four centromeres, two for eac homologous chromosome.

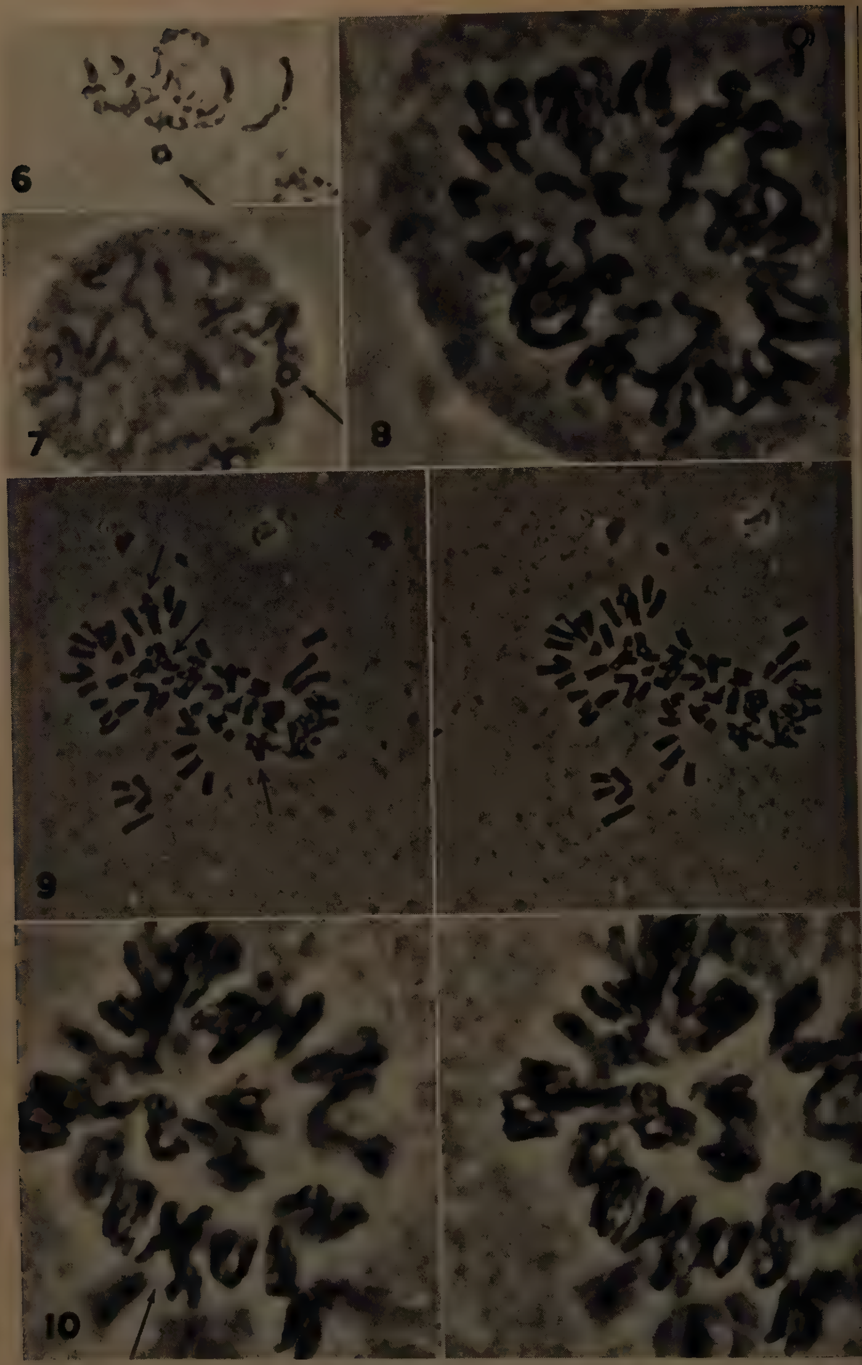
The outstanding characteristic of the centromeres, as revealed in th *Drosophila* study, is their lack of chromosomal specificity. All centromer synapse into a single mass and are therefore homologous; no other structures of the several chromosomes show such homology or synaptic tendency. Chromosomal structures having this feature of homology and synapsis were now sought in the germinal cells of the mouse and were found without difficulty. In pachytene nuclei, whose chromosomes are not completely separated in the squash method of preparation, the chromosomes are found to be associated at random, in groups of two to six, all of the members of each group radiating from a common central point that consists of closely synapsed prominent chromomeres. Typical examples are seen at the top of FIGURE 2, which is taken from one of the working drawings. By analogy with the similar structure of *Drosophila*, these chromomeres are assumed to be the centromeres.

The phase-contrast map was now revised on the basis of having each map element begin with its centromeres. Twelve of the twenty chromosomes have now been completed and are found to be reliable as the basis for chromosome identifications. Through their use, four of the larger chromosomes, III, V, VIII, and XI, can be identified in prophase nuclei of embryonal cells in the teratoma. Upon completion of the remaining eight map elements, the thorough analysis of tumor nuclei should be possible. Indications of the role of chromosomal hypoploidy, hyperploidy, and major aberrations in tumor biology will be sought.

Strong indications have been presented elsewhere in this monograph by Biesele and by Levan and Biesele that nuclear changes may precede the transformation of normal cells into malignant or potentially malignant cells. The following account will indicate clearly that nuclear changes do not cease after the onset of malignancy, and the cytogenetic mechanisms involved are elucidated in several instances.

The formation of interpolar bridges at the anaphase stage (FIGURES 3, 4, and 5) is a commonly observed phenomenon in tumor cells. In embryonal cells of the teratoma previously mentioned, such bridges were found to occur at a high frequency in various generations throughout the transplantation history of the tumor. For example, ascitic transplant generations 90, 97, 103, 117, and 131 showed bridges in five to twelve per cent of all anaphases observed. Further study revealed the presence in generations 97 and 117 of a ring-shaped chromosome not detected in previous or subsequent generations, and easily discerned in metaphase plates (FIGURES 6, 7). Since ring chromosomes, as classically known from both *Drosophila* and corn studies, are producers of frequent bridges of both interlocking and dicentric types, the tumor nuclei are seen to contain a chromosomal aberration capable of providing a means for bridge formation and chromosome eliminations. Hauschka[6] has also found ring chromosomes in tumor cells.

In addition to its rings, generation 97 contained a second type of aberration. In the metaphase plate of a single tetraploid cell, a typical inversion figure was found (FIGURE 8). This figure is of considerable interest since it appears in a cell in which chromosomal synapsis is not present. It is formed within a pair of sister chromatids, and is seen in the actual cell in which the aberration occurred. If an exchange of strands could have taken



FIGURES 6 to 10

place within the limits of the inversion, the resulting acentric and dicentric portions would have been eliminated in the subsequent anaphase.

These gross types of aberration, while cytogenetically important in themselves, have their greatest significance in tumor biology as indicators that chromosome breakages and reorganizations are of frequent and perhaps constant occurrence. Equally good indicators of reorganization are those that fall into the category of exchanges of linkage—the translocation family. Whether between nonhomologous chromosomes (translocations) or between homologues (crossovers, perhaps both equal and unequal), linkage changes provide a means for modifications of the genome. The changes may be drastic, as in the case of segmental aneuploidy resulting from aberrant separations of translocation members; or they may be subtle modifications, such as the segmental homozygosis resulting from the segregation of crossover members. Three examples of segmental exchange are indicated by arrows in FIGURE 9, a stereoscopic photograph of a hypertetraploid metaphase in an embryonal ascites cell of the teratoma. The arrow in stereoscopic FIGURE 10 indicates an exchange between two members of a synaptic tetrad in a tetraploid metaphase of the same material. It is probably significant that such exchanges, whether translocations or crossovers, have thus far been observed only in tetraploid cells in the 139 ascitic transplant generations of the tumor; synapsis and tetrad formation are likewise confined to tetraploid cells.

The role of mutations—of all types and sources—in tumorigenesis has been discussed by Burdette[7] in his splendid review of the topic. In the present paper it is necessary to point out only that chromosomal aberrations are reliable indicators of gene mutation, at least in the position-effect category, and that cytogenetically explainable chromosome aberrations are especially prominent in malignant cells; consequently it can be concluded that the frequency of concomitant mutations is also high. Considered together, these nuclear changes are sufficient to explain the capacities for variation within tumor lines; even omitting all mutation phenomena, the chromosomal losses, interchanges, and segregations may be sufficient in that they modify established systems of genic balance within the cell. Since genic balance determines all gene attributes, including mutability and dominance, the elucidation of balance phenomena in both normal and malignant cells is the major responsibility of cytogenetics in the immediate future. It can be assumed with considerable conviction that, among mammals, only the mouse shows promise of becoming sufficiently well known genetically to permit the establishment of a direct relationship between malignancy and single genes, groups of genes, or whole chromosomes.

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GENETIC PLANT TUMORS IN *NICOTIANA*[*]

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Genetic tumors are those that arise spontaneously in particular genotypes without being incited by any detectable environmental agent, and the tumor condition is hereditary. They have been observed to occur throughout the plant and in entire progeny populations of 28 different hybrid combinations of species of *Nicotiana*.[1] In 23 additional species combinations genetic tumors have been reported to be produced irregularly in some of the progeny or restricted to only certain parts of the plants. Species of the Alatae section of the genus,[2] most frequently *N. langsdorffii*, are frequently involved as one of the parents. The tumor-forming hybrids comprise only a fraction of the total number of *Nicotiana* species combinations that have been produced; for example, Kostoff[3] listed 181 different F_1 hybrids, 69 reciprocals of them, 15 amphidiploids, and 5 crosses between amphidiploids, making a total of 270 species combinations. Studies on the occurrence, cytology, morphology, physiology, and tissue culture of *Nicotiana* tumors have been made since the 1930's.[3-9] The hybrid and amphidiploid *N. glauca-langsdorffii* has been studied most extensively. It has been shown[1, 3, 8] that tumor induction is not transmitted across the union of reciprocal grafts between this *Nicotiana* hybrid and related nontumor-forming types (other *Nicotiana* species and tomato), thus providing experimental evidence that a transmittable tumor-inciting virus is not involved. Black[10] has pointed out difficulties in disproving viruses as causal agents.

Histologically[11-13] the tumors range from smooth nonorganoid masses with diffuse groups of dividing undifferentiated cells to leafy or stalked types with aberrant leaf primordia and considerable vascular development (FIGURE 1). Tumor formation is usually initiated in parenchymatous cells in the region from the pericycle to the outer cortex.

Physiological studies have revealed that rapidly growing tumor tissue compared with controls of parental or nontumorous tissue show fourfold to sixfold increases in the amides glutamine and asparagine, and moderate increases in aspartic acid, glutamic acid, and glycine.[13] The protein synthesized in a variety of randomly proliferating plant tissues, including that of *N. glauca-langsdorffii* tumors, contains hydroxyproline, which rarely occurs free or combined in plants.[14] The hydroxyproline has been shown to arise from proline in the protein or at the point of synthesis.[15] The tumor-forming amphidiploid is higher in free auxin, possesses a more effective enzyme system for conversion of tryptophan to auxin, and has a higher free tryptophan content than either diploid parent.[1] Coconut milk and malt and yeast extracts[16, 17] induce tumors in tissue cultures of normal diploid species of *Nicotiana* and have a stimulative action on tumor culture. Manifestation

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[*] The research described in this paper was carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

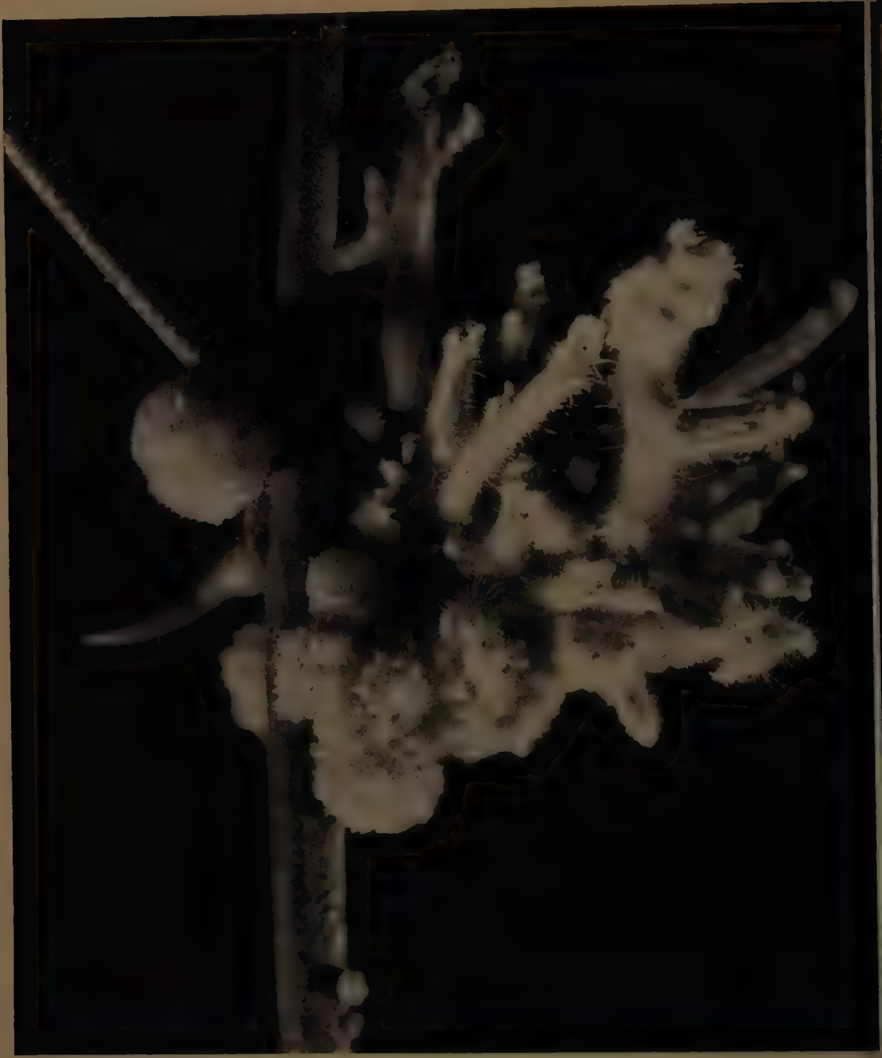


FIGURE 1. Spontaneous genetic tumors formed in the region of a node on the stem of the amphidiploid *Nicotiana suaveolens-langsdorffii*. Relatively undifferentiated tumors are shown to the left, differentiated tumors to the right.

of organ formation versus undifferentiated growth in tumor cultures can be modified by altering the media or method of culture. Organ formation is enhanced by addition of adenine, phosphate, and sucrose; by low concentration of indoleacetic or naphthalene acetic acid; and by conditions of low light intensity, a temperature of 18° C., and a liquid medium.[1, 18] Scopoletin has been found to be associated with differentiation of structures in a morphologically distinct strain of nongenetic tumor of *Nicotiana affinis*.[19] In conclusion, the physiological investigations have demonstrated biochemical differences in content and in cultural requirements of tumors, in contrast to normal tissue. However, the causes of the metabolic and morphological change leading to tumor initiation remain obscure.

The effect of ionizing radiation on tumor formation in *N. glauca-langsdorffii* has been described in a number of recent papers.[13, 20-26] These investigations demonstrated an enhancement of tumorous growth correlated with dosage of radiation, so that tumors developed earlier and comprised a progressively larger proportion of the total plant weight with exposure to increasingly greater amounts of irradiation. The same phenomenon is exhibited by the amphidiploid *N. suaveolens-langsdorffii*, according to our observations.

Cytogenetic studies have shown that tumors form on any diploid, triploid, or tetraploid combination of *N. glauca* and *N. langsdorffii* that possesses at least one complete genome of each of the parent species.[1] In an effort to establish an association of tumor formation with particular chromosomes, the amphidiploid *N. glauca-langsdorffii* was backcrossed to *N. langsdorffii* and the backcrossing was then continued, accompanied by selection for extra alien chromosomes of *N. glauca*. However, no progeny with less than a full genome of *N. glauca* combined with *N. langsdorffii* produced tumors.[1] This result provided additional evidence of genotypic control of tumor initiation, but precluded further chromosomal analysis of this sort. French investigators have reported that, among the progeny of plants produced from X-ray-treated germinated seeds of *N. glauca-langsdorffii*, nontumoral types were obtained.[27-29] They concluded from a study of reductional prophases in certain lines that the character "absence of tumors" is probably associated with a loss of chromosomes. This result is compatible with the results of our backcrossing experiment described above. More recently, however, Izard[30] described two tumorless lines derived from X-rayed seed as above, which have the normal amphidiploid complement of 42 chromosomes, regular meiotic divisions, and 80 to 100 per cent good pollen. This is supporting evidence for the gene control of tumor formation.

Kostoff reported[3, 31] an increase in frequency of abnormal mitoses in tumor tissue, but a causal relation to tumor initiation appears unlikely in view of the observation that chromosomal loss is associated with reduction or absence of tumor initiation. Somatic exchanges between heterogenetic chromosomes could conceivably be causal to tumor initiation. Polyploidy is not causally involved, since F_1 hybrids and their amphidiploids show the same tumor-forming properties. Inequalities in transmission of cytoplasmic elements do not influence tumor formation, since tumors of reciprocal hybrids are identical.[3, 32]

Materials and Methods

The basic plant material used in these experiments is the amphidiploid *Nicotiana suaveolens-langsdorffii*, which produces genetic tumors similar to those of *N. glauca-langsdorffii*. It has certain advantages, such as earlier maturity and greater ease of hybridization. In order to explore the relation between tumor initiation and somatic mutation, an attempt was made to synthesize plants on which both phenomena could be observed simultaneously. *N. suaveolens-langsdorffii* was crossed with pollen of *N. sanderae* which is homozygous for a basic dominant color gene, *P*, the presence of which permits development of anthocyanin pigmentation in the corolla. The resulting hybrids (FIGURE 2) were vigorous plants with solid red (*Pp*) flowers

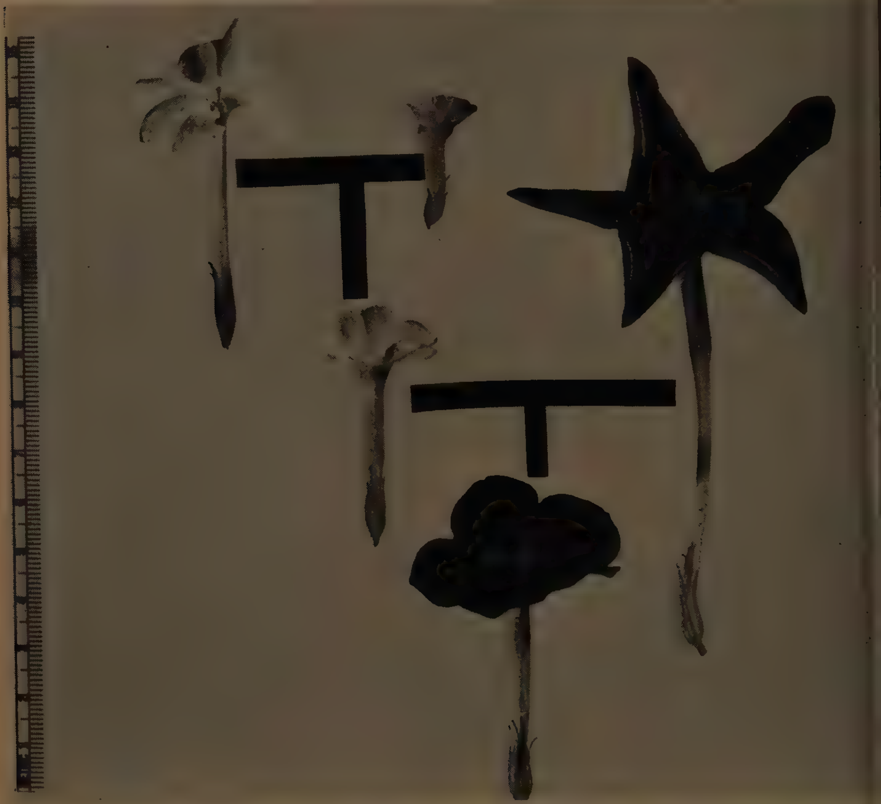
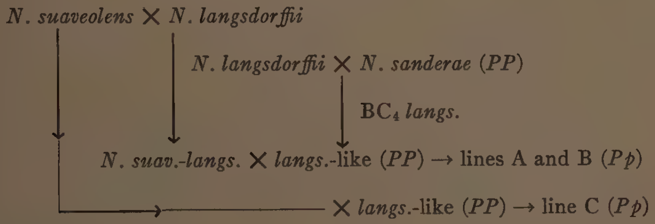


FIGURE 2. Flowers illustrating hybridizations made between species of *Nicotiana* Upper: left, *N. suaveolens;* right, *N. langsdorffii.* Center: left, amphidiploid *N. suaveolens-langsdorffii* (forms tumors); right, *N. sanderae* (basic color gene, *PP*). Lower: hybrid between *N. suaveolens-langsdorffii* and *N. sanderae* (no aerial tumors).

They were grown in a control field and under conditions of chronic gamma radiation (10 to 400 r per 20-hour day) in the summer of 1956. Unexpectedly, no tumors were formed on the aerial parts of these plants in spite of the presence of a full genome of *N. suaveolens* and of *N. langsdorffii.* The addition of a genome of *N. sanderae* afforded "protection" against tumor formation. It should be noted that the interspecific hybrid *N. suaveolens* × *N. langsdorffii* forms tumors, whereas the interspecific hybrid *N. suaveolens* × *N. sanderae* does not.

In order to retain the tumor-forming property of *N. langsdorffii* combined with the basic color gene of *N. sanderae*, the interspecific hybrid between them was backcrossed 4 times to *langsdorffii* accompanied by selection for full anthocyanin pigmentation. In one of these lines a plant was selected and crossed as a pollen parent to *N. suaveolens-langsdorffii*. This was the origin of line A, which was grown in the summer of 1957 under the culture number 57163. In another backcross line of similar origin a single plant was selected and crossed as a pollen parent to the same amphidiploid and to the species *N. suaveolens* (FIGURE 3). These cultures were designated as line B (57162) and line C (57164), respectively. The derivation of these lines is shown in the accompanying diagram.


N. suaveolens × *N. langsdorffii*
N. langsdorffii × *N. sanderae* (*PP*)
BC_4 *langs.*
N. suav.-langs. × *langs.*-like (*PP*) → lines A and B (*Pp*)
× *langs.*-like (*PP*) → line C (*Pp*)

The 3 lines—A, B, and C—were planted as rosette-stage plants on June 18 in a control field and at 9 different distances from the cobalt-60 source in the gamma field. The radiation dosages at the locations used were 1.24, 2.5, 5, 10, 25, 50, 100, 150, and 200 r per 20-hour day. The upper limit of 200 r per day was chosen, since previous experiments had shown that this dosage would be sufficient to cause a large increase in mutation rate, yet would not produce malformations of the flower, which interfere with scoring somatic mutations, or marked injury of apical meristems, which stimulates tumor formation.

Somatic mutations were scored during the last 10 days of July by noting the number of white (*p*) sectors (FIGURE 4) in an area one fourth of a centimeter square in the same location on each of the 5 petals.[26] In general, 10 flowers were classified at the higher dosage levels (50 to 200 r) and 30 flowers at the low dosages (control to 25 r). The total number of white sectors for the 5 petal areas were added together, the sum was divided by the number of flowers scored, and this mean was multiplied by four fifths to give the average number of mutations per square centimeter at each treatment for each of the 3 lines. This constituted the data on mutation frequency. It should be emphasized that the term "mutation" is used in a broad sense and merely indicates loss of color attributed to loss, inactivation, or alteration of the dominant *P* allele. In all probability it usually reflects loss of the chromosome region bearing the *P* locus. Its intended use in the context of this paper is as a quantitative measure of the influence of radiation on genetic material.

During late August and early September the same plants that had been scored earlier for mutation frequency were classified for frequency of aerial

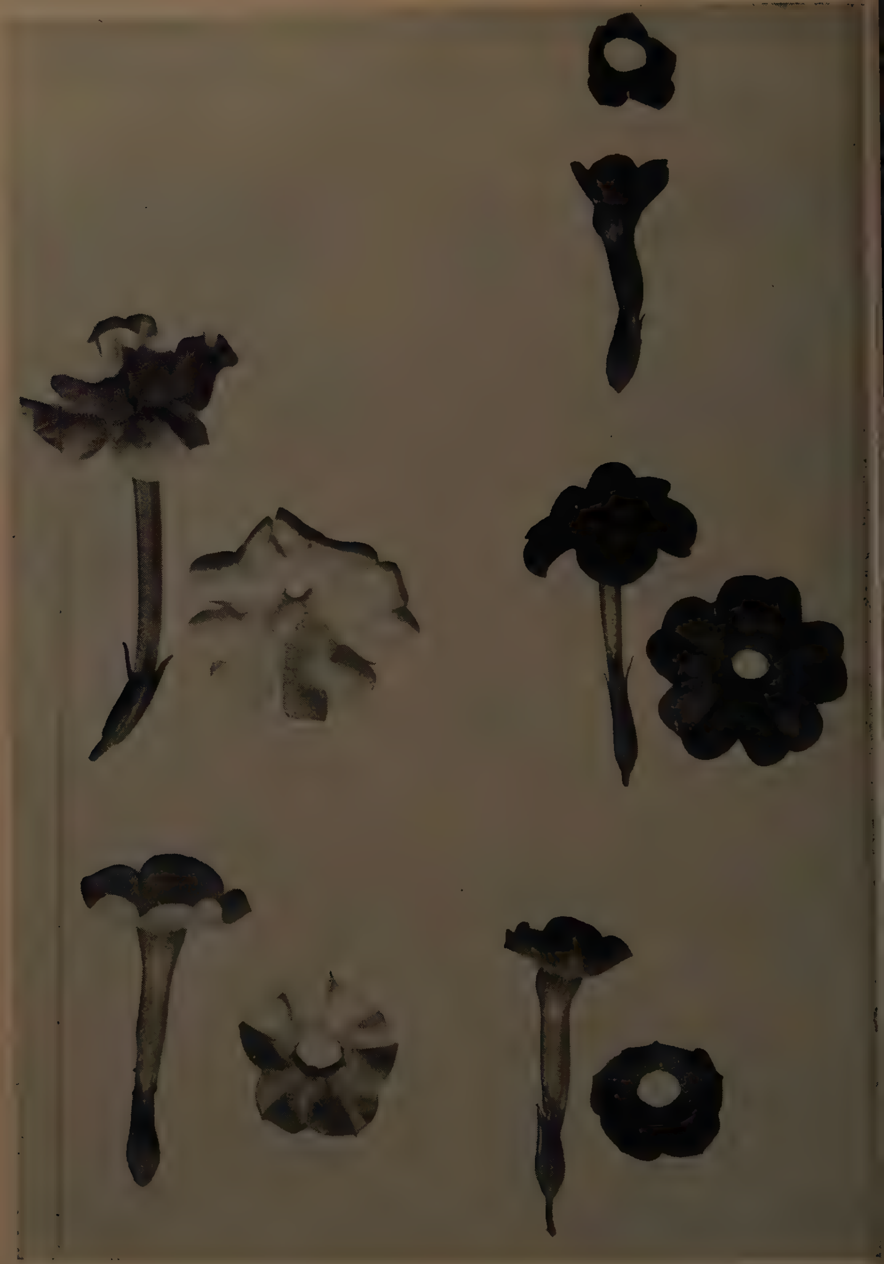


FIGURE 3. Flowers illustrating origin of two lines of *Nicotiana* synthesized for tumo production and scoring "somatic mutation." Left: upper, *N. suaveolens;* lower, *N. suaveolens-langsdorffii*. Right: upper, selection with color of *N. sanderae* on a genotypic background that is preponderantly *N. langsdorffii;* center, line C formed by crossing the selection with *N. suaveolens;* lower, line B formed by crossing the selection with *N* *suaveolens-langsdorffii.*

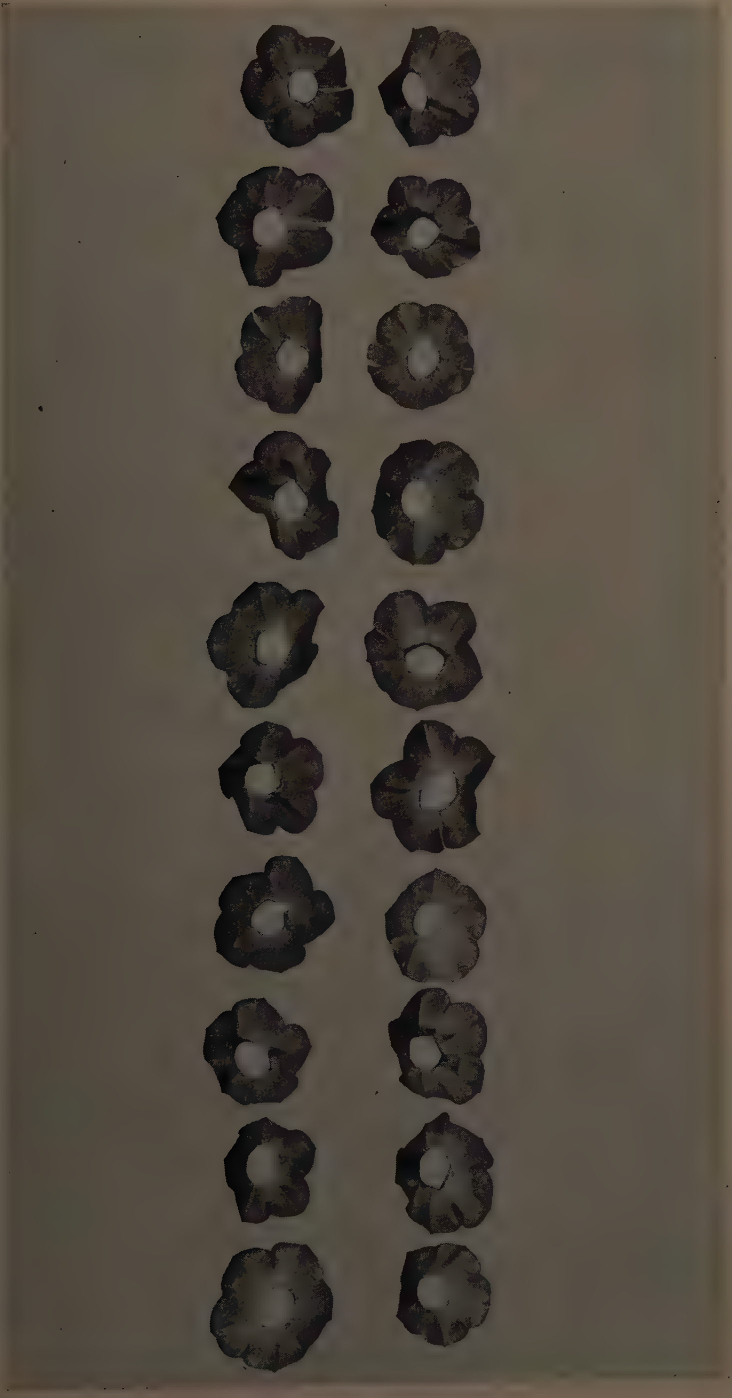


FIGURE 4. Flowers of line A (above) and line B (below) showing control (left) and those exposed to increasing dosages of chronic gamma radiation from 1.24 (second from left) to 200 (extreme right) r per 20-hour day. White sectors, owing to loss of *P*, were scored as "somatic mutations."

tumors (FIGURE 5). These tumors were generally smaller than on the amphidiploids *N. glauca-langsdorffii* and *N. suaveolens-langsdorffii*. Particularly in lines A and B (hybrids with the amphidiploid) proliferations tended to form almost exclusively at the nodes and to remain discrete. Observations made on the primary stem, 3 secondary branches, and 12

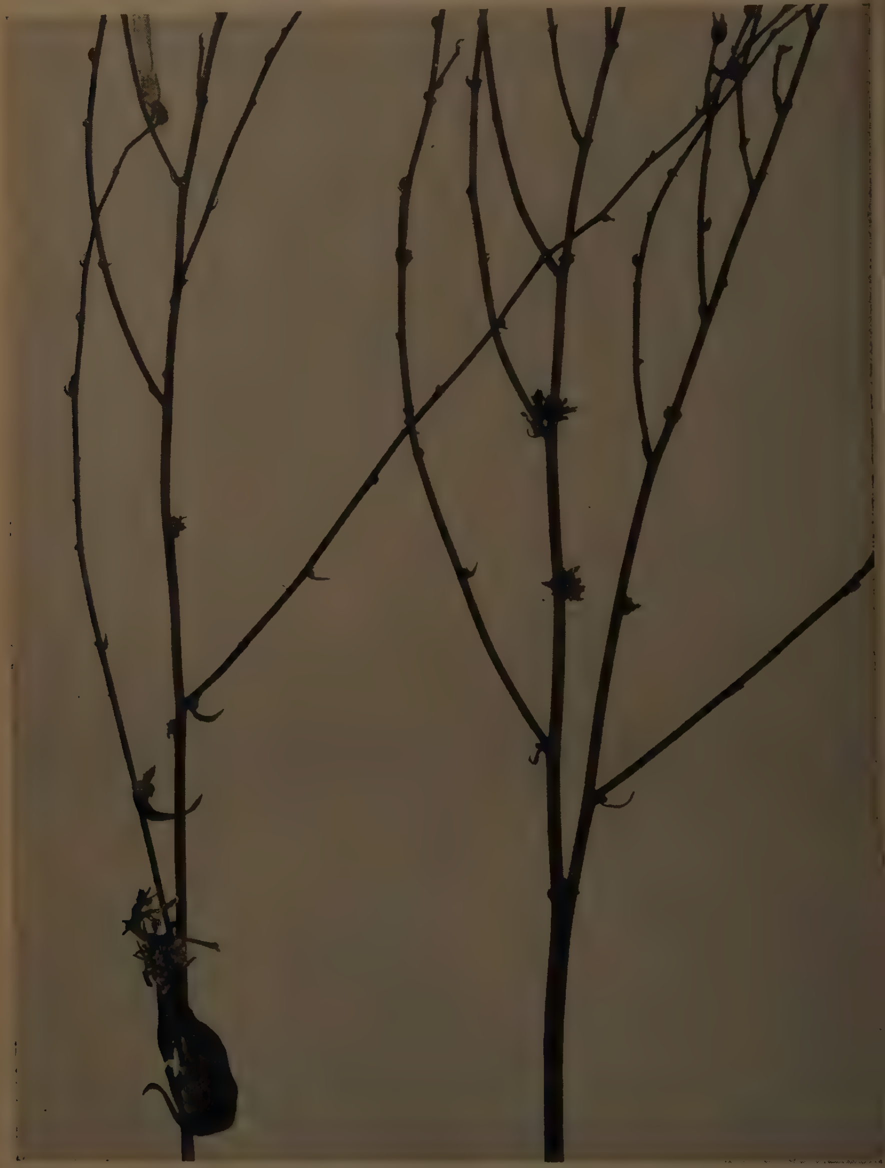


FIGURE 5. Stems of synthesized line A showing small discrete, primarily nodal tumors, which were scored to obtain data on frequency of tumor initiation.

tertiary branches of each plant were handled separately, and the data were expressed as the percentage of nodes having tumors. Greater emphasis was placed on obtaining data from the younger branches, since they normally produce fewer tumors and would therefore be expected to provide better material for observing effects of radiation.

Four additional cultures were included in these investigations. They consisted in crosses, the male parents of which were derived from the hybrid *N. langsdorffii* × *N. sanderae* backcrossed four or seven times to *N. sanderae*, accompanied by selection for specific marker genes of *N. langsdorffii*. The female parents were *N. suaveolens* and *N. suaveolens-langsdorffii*. The cultures were as follows.

57166: *N. suav.-langs.* × *N. sand.* (BC$_7$) with *langs.* genes *r* and *e*.
57167: *N. suaveolens* × *N. sand.* (BC$_7$) with *langs.* genes *r* and *e*.
57168: *N. suaveolens* × *N. sand.* (BC$_4$) with *langs.* genes i_1, i_2, and *D*.
57169: *N. suaveolens* × *N. sand.* (BC$_4$) with *langs.* genes *p* and *G*.

They were grown only in the control field and were examined in only a qualitative way for tumor formation.

Results

"*Somatic mutations*." The frequency of "somatic mutations" expressed as the mean number of white sectors per square centimeter of corolla petal surface is shown in TABLE 1. Ninety-five per cent confidence limits around the average values are represented in the table as twice the standard error of the mean. Clearly, there is an increase in mutation frequency with

TABLE 1
FREQUENCY OF SOMATIC MUTATIONS IN THREE LINES OF *Nicotiana* EXPOSED TO CHRONIC GAMMA RADIATION

Treatment	A 57163			B 57162			C 57164		
	n	$\bar{x} \pm 2s_{\bar{x}}$		n	$\bar{x} \pm 2s_{\bar{x}}$		n	$\bar{x} \pm 2s_{\bar{x}}$	
Control...	30	0.48 ± 0.19		30	0.22 ± 0.14		30	0.38 ± 0.26	
1.24 r.....	30	0.64	0.30	30	0.40	0.24	30	0.46	0.21
2.5 r......	30	0.98	0.35	30	0.24	0.16	30	0.48	0.21
5 r........	30	1.18	0.37	30	0.78	0.27	30	1.06	0.42
10 r.......	30	2.24	0.45	30	2.08	0.67	30	2.58	0.56
25 r.......	30	4.06	0.51	30	4.26	0.64	30	5.78	0.80
50 r.......				10	10.20	2.40	10	13.50	2.20
100 r......	10	13.00	2.10	10	17.60	2.10	23	24.60	2.40
150 r......	20	20.80	2.00	60	27.70	1.30	30	28.60	2.20
200 r......	10	26.90	1.90	10	41.40	4.50	17	47.30	3.00

increasing radiation dosage. The average increase in mutation rate at 200 r over the control rate in the 3 lines is 123-fold.

In FIGURE 6 the data on number of mutations per sq. cm. of petal are shown plotted against r dosage of chronic gamma radiation per 20-hour day. The relationship is clearly linear in culture A. For lines B and C there is some indication of nonlinearity; however, in all three lines mutations of the same allele, *P*, are being scored. Assuming linearity, the increase in number of mutations per sq. cm. for each increase in one r per 20-hour day is 0.134, 0.186, and 0.215 in cultures A, B, and C, respectively. Analysis

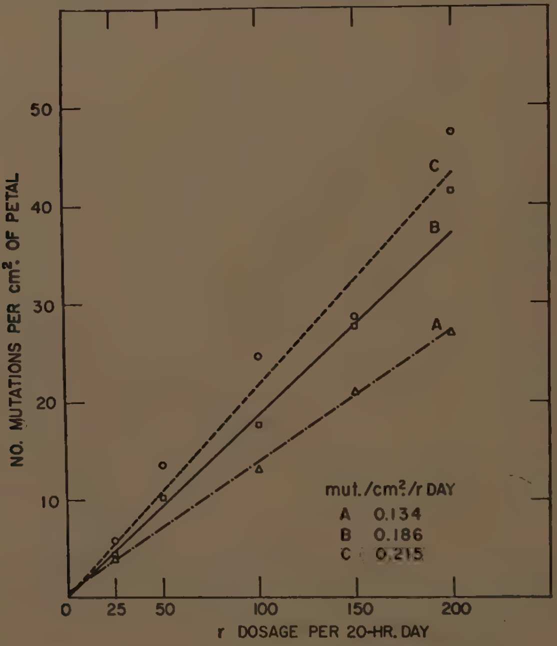


FIGURE 6. Regression lines showing the relation between frequency of "somatic mutations" and r dosage of chronic gamma radiation per 20-hour day. The calculated slopes show the magnitude of increase in mutations for an increase in one r unit.

indicates that three separate regression lines fit the data significantly better than a single regression line; hence it is concluded that the three cultures respond differently to radiation. Since the same allele is being scored in each culture the results therefore constitute evidence that the genotypic background—which is different in each culture—is responsible for the different responses of the *P* allele to chronic gamma irradiation.

Tumor formation. Data on tumor formation expressed as the percentage of nodes with tumors is summarized in TABLE 2 for 3 ages of stems in each of the 3 synthesized lines. There was an over-all correlation between age of stems and tumor frequency in that, on the primary stem, 81 per cent of the nodes were tumorous; on the secondary branches, 76 per cent; and on the tertiary branches, 62 per cent. There was no obvious trend or correlation between radiation dosage and frequency of nodes with tumors. The relationship was tested statistically by obtaining the ratio of the variance between treatments over the variance within treatments (controls and 150 r). None of these ratios was significant, as shown in TABLE 3. Clonal populations, the use of which might have reduced the variance within treatments, were not

TABLE 2

FREQUENCY OF NODAL TUMORS IN THREE LINES OF *Nicotiana* EXPOSED TO CHRONIC GAMMA RADIATION

Treatment	Percentage of Nodes with Tumors								
	Primary stem			Secondary branches			Tertiary branches		
	A	B	C	A	B	C	A	B	C
Control 1...	83.3	81.0	100.0	70.8	84.6	94.8	60.0	75.6	88.4
2...	85.7	56.4	100.0	65.7	62.5	87.2	35.1	57.4	59.0
3...	76.7	95.7	75.9	92.3	87.2	71.8	76.5	75.0	65.1
x̄...	81.9	77.7	92.0	76.7	78.1	84.6	57.2	69.3	70.8
1.24 r......	60.7	100.0	83.3	66.7	61.3	63.4	45.6	46.4	68.9
2.5 r.......	80.8		56.3	57.1		71.7	55.5		56.0
5 r.........		83.9	77.8		76.3	75.0		57.7	65.4
10 r........	69.0		65.7	68.1		73.9	56.1		60.2
25 r........	90.0	89.3	85.7	85.7	76.7	73.0	59.4	66.0	61.5
50 r........		84.0	85.2		87.2	85.1		69.2	67.8
100 r.......	70.6	79.3	100.0	51.1	74.0	75.0	44.6	73.1	55.3
150 r.......	63.2	90.5	81.3	82.9	86.3	88.9	47.1	70.6	65.4
150 r.......	85.2	76.5		79.6	72.7		60.8	77.2	
200 r.......	81.8	83.9	88.5	69.0	85.1	91.4	58.4	69.5	75.2
x̄ r.........	75.2	85.9	80.4	70.0	77.5	77.5	53.4	66.2	64.0
Over-all x̄...	77.0	83.7	83.3	71.7	77.6	79.3	54.5	67.1	65.7
		81.4			76.3			62.5	

TABLE 3

VARIANCE RATIOS FOR FREQUENCY OF NODAL TUMORS IN THREE LINES OF *Nicotiana* EXPOSED TO CHRONIC GAMMA RADIATION

Line	Variance ratios, F			$F_{.05}$
	Primary stem	Secondary branches	Tertiary branches	
A	1.00	1.19	0.11	8.88
B	0.21	0.41	1.22	8.88
C	0.93	0.63	0.20	19.38

available for these studies. It was concluded that there was no significant difference in tumor initiation among radiation dosages in any of the 3 lines or in any of the 3 ages of stems. There was no significant regression of tumor initiation on radiation dosage as measured in r units per 20-hour day of chronic exposure (FIGURE 7).

It is equally evident, therefore, that there was no correlation between frequency of somatic mutation and frequency of tumor initiation. A range of radiation dosages that caused a more than one hundredfold increase in mutation frequency of the *P* allele had no detectable influence on initiation of tumors (FIGURE 7) in this material. The basic color gene is heterozygous

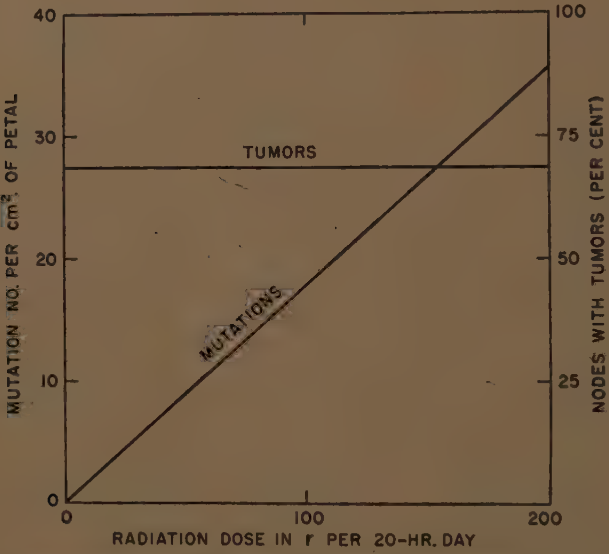


FIGURE 7. Regression lines of frequency of somatic mutations and of nodal tumors on r dosage, showing correlation with the former and lack of relation with the latter. Combined over-all data were used in constructing the graphs.

in all three lines; the factors controlling tumor initiation are all heterozygous in line C and, at least as far as the *suaveolens* component is concerned, in lines A and B as well. Conceivably, tumor initiation could be due to an unstable system in which somatic mutation events are insensitive to the dosages of radiation employed. Demerec[33] observed that reversions in the unstable miniature-3 gene of *Drosophila virilis* were not significantly affected at X-ray dosages that increase the general rate of mutations about 1000 per cent. On the other hand, an unstable locus in a derivative of the hybrid *N. langsdorffii* × *N. sanderae* was found to be more sensitive to low dosages of radiation than was a stable locus.[34]

Another line of evidence that appears to oppose the somatic mutation hypothesis of tumor initiation derives from the sexual progeny of tumors. Stemlike protuberances extending from tumor masses (FIGURE 1) may occaionally elongate into thickened stems and produce flowers and seed. The hickened stems are morphologically unique and are characterized by a “wavy” vascular system (observation courtesy of Otto L. Stein).* Seed rogeny from these aberrant branches differed in no observable way, including tumor formation, from seed progeny derived from normal stems.

Segregation for tumor formation. Two lines, with a genotype preponder-ntly of *N. sanderae* (fourth backcross) in which specific genes of *N. langs-orffii* were maintained by selection, were crossed with *N. suaveolens.* A few iable seeds were produced. Two surviving hybrid plants from the cross 7168 involving *sanderae* + *langsdorffii* genes i_1, i_2, and *D* failed to form umors. Of 34 progeny from the cross 57169 involving *sanderae* + *langs-orffii* genes *p* and *G*, one plant produced a single large tumor, another plant ad several large tumors in the inflorescence, and the remaining 32 plants howed no evidence whatsoever of tumors. This was the most clear-cut vidence of segregation for tumor-forming tendency to appear in our cultures. A third line of preponderantly *sanderae* genotype (seventh backcross) with *angsdorffii* genes *r* and *e* was crossed with *N. suaveolens* and *N. suaveolens-angsdorffii*, giving 3 surviving plants in the former (57167) and 23 in the atter (57166) culture. All plants in 57166 produced tumors that characteristically appeared only at nodes, had considerable purple pigmentation, and vere relatively highly differentiated, consisting largely of stemlike growths esembling witches'-broom. Some solid masses of tumor were as much as 2½ inches in diameter. Of the 3 plants in culture 57167, one produced everal definite tumors, while the other 2 formed occasional swollen nodes vith excessive branching, but no unequivocal tumors.

These preliminary results indicate that through recombination of genes of *V. langsdorffii*, which incites tumor formation when combined with *N. uaveolens*, and *N. sanderae*, which inhibits the inherent tumor-forming uality of *N. suaveolens-langsdorffii*, segregation for markedly different egrees of tumor expression can be expected. With this in mind 65 pollinaions of the cross *N. suaveolens* by F_1 (*langsdorffii* × *sanderae*) were made. Of 13 viable hybrids produced, 5 formed tumors and 8 did not. This result rovides clear evidence of segregation for tumor formation. Of potential nterest are hybridizations using as pollen parents the F_1 and F_2 segregants of *V. langsdorffii* × *N. sanderae*, and as seed parents *N. suaveolens-langsdorffii*, *V. glauca*, *N. glauca-langsdorffii*, and *N. suaveolens-glauca*.

Summary

Spontaneous tumors occur in the genus *Nicotiana* only in certain species ombinations, and their expression may be modified by altering the chromoomal and genic composition of the hybrids. In this sense the tumors are enetically controlled. Insofar as can be generalized from the experimental esults reported here, the initiation of tumors in plants genetically contituted to produce them is causally unrelated to somatic mutation; that is, demonstrably effective dosages on a marker gene of the mutagenic agent gamma radiation, did not increase the frequency of tumor initiation.

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Braun's investigations[35] indicate that the tumor-inducing principle in tobacco crown gall initiates a gradual change to the autonomous production of greater-than-regulatory amounts of a cell-enlargement factor or auxin a cell-division factor or kinetin, and certain essential amino acids and vitamins. In genetic tumors this inducing principle, possibly itself a DNA,[3 may be autonomously synthesized and quantitatively controlled by the action of interacting genes contributed by both parental species (see Kehr's discussion of Braun's paper[35]).

Biochemical differences that have been found between normal and tumorous tissue provide a basis for further investigations on the metabolic disturbances[32, 37] (apparently accentuated, but not initiated, by ionizing radiation[25]) that lead to undifferentiated growth. Genotypes synthesized so as to segregate for different manifestations of tumor formation offer nove material for a biochemical-genetic approach to the problem.

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Discussion of the Paper

DONALD F. JONES (*The Connecticut Agricultural Experiment Station an* *Yale University, New Haven, Conn.*): The evidence presented in this publication and elsewhere shows clearly that normal growth and development resul from the interaction of genes, cytoplasm, and environment, both externa and internal. This interaction is dependent upon a well-balanced syste resulting from countless generations of selection under all possible environmental conditions.

Drastic changes in this balanced system may result from many sources the chief of which can be recognized in gene alteration, chromosomal rearrangement, and virus contamination. Plants are especially favorabl material in which to show how these changes may be brought about. Plant have cell walls; many gene products are not diffusible through these cel walls, so that changes initiated in the nucleus are visible only in the cytoplasm of the cell in which they originate and in the daughter cells derive from this altered cell.

Nuclear changes affecting growth appear spontaneously in both plant and animals and are clearly associated with chromosomal rearrangement as shown by the visible changes in gene markers, resulting in paired o unpaired sectors with changes in color, chemical composition, or cell siz and arrangement easily visible under a low-power microscope.[1] Thes changes may or may not be accompanied by polyploidy or gross chromosoma aberrations. The evidence indicates that these irregularities in chromosom behavior are the result and not the primary cause of changes in growth.

Obviously, we need to know more about the normal control of growt before we can hope to control or prevent abnormal growth. There is goo evidence that change in the nucleus-cytoplasm relation is the beginning o species separation.[2] There is much in common between species formatio and the changes that bring about irreversible and uncontrolled growth. I both cases there is the loss of control, and genes of one type fail to functio normally in the cytoplasm of the other type. The failure to note an maternal effect on tumor formation in the species hybrids reported b Harold H. Smith and others may be due to elimination in seed-propagate progenies. In somatic changes this elimination is not involved unless th changes are cell-lethal. Many vegetatively propagated varieties of horticultural plants result from wide crosses, and it is noteworthy that many o these show much pollen and ovule abortion, as well as chlorophyll variegation. These visible characters are the first indication of a failure of a norma genome-plasmon-environ relation.

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ENETIC CONSIDERATION OF LEUKOSIS TUMOR GROWTH IN POULTRY[*]

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The chicken is the host for a cell-proliferating disease having many synromes, lymphoid, erythroid, neural, myeloid, and even osteoid types, comionly called the leukosis complex (Jungherr, 1948). The tumor charactertics are so diverse as to call for their initiation by several different agents, et the existence of more than a single agent is yet to be proved. There is ir evidence that a virus is associated with the tumor formation, for filterable xtracts under well-controlled conditions appear to initiate the disease. isease syndromes characteristic of particular tumors are transmitted from hick to chick by inoculation of tumor cells. This tumor material preumably contains a viruslike entity, as well as the living tumor cells. Ordiarily, the filtrate alone takes some weeks or months to progress from infection o tumor formation. Inoculation of separated tumor cells, on the other and, takes less than two weeks. The lapsed time between tumor inocuation and the overt tumor disease favors the view that in tumor inoculation is the tumor cell growth potentials that are being evaluated rather than ny virus properties the cells may contain.

In our studies of the leukosis-chicken relationship my associates and I ave chosen to work with tumor cells of a single tumor line, Research Poultry aboratory (RPL) 12, as genetic interest increases where both infectious gents are involved. The attack on the problem is four-phased. Host enetic differences in resistance or susceptibility to the tumor implants have een sought. The tumor cells have genetic constitutions that may differ; ome are favorable to invasion of one host, some are of lesser virulence. ontact with the tumor cells may lead to host reactions that may affect the umor infectivity. These reactions may be of the humoral type or they ay be the consequence of more direct cellular action on the part of the host Gowen, 1948, 1952, 1954). Studies on the first of these problems were made y R. P. Poirier and myself and were published as theses at Iowa State ollege (Poirier, 1951, 1952). Later studies are by Eugene C. Seu, William . Haensly (Haensly, 1956), and myself (Gowen, 1937–1954). The Iowa ivision of the American Cancer Society, Inc., cooperated in the support f the research.

The Host Factor in Leukosis

The following experiments were designed to ascertain if chickens, as hosts f leukosis, have a range in naturally inherited susceptibilities and resistances o leukosis tumors derived from the RPL tumor 12.[†]

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[†] This tumor was received by us from the Regional Poultry Research Laboratory, nited States Department of Agriculture, East Lansing, Mich., in a frozen state and aintained by us as transfers through successive control chickens.

Two hundred sixty-two families of chickens having 7 to 12 progeny wer inoculated into the breast muscle with a standard dose of virulent tumo suspension. The families varied in survival from 0 to 100 per cent. Fro these results highly resistant families were chosen on the one side and highl susceptible families on the other. Further progeny were obtained fro these families. The progeny that were separated out of this initial popu lation on the basis of these tests became the foundation stocks for ou resistant and susceptible strains. The average results for the breeds o poultry from which the families came are shown in TABLE 1, which show that there are breed differences in survival, but that the differences are, o the whole, small. The breeds give the impression of being representativ of poultry in general. Each surviving chick was inoculated twice wit 0.25 cc. of a mixture of tumor cells and saline in the proportions of 1 to 4

TABLE 1

BREEDS OF CHICKENS AND AVERAGE DEATHS FROM INOCULATED LEUKOSIS TUMORS FOR FOUNDATION FAMILIES

Breed	Number tested	Per cent survived
White Leghorn	1496	33.0
Rhode Island Red	242	35.5
White Plymouth Rock	116	25.9
New Hampshire	66	50.0
Black Minorca	53	45.2
White Minorca	23	21.7
Total	1996	Average..... 33.7

Seven hundred and seventeen chicks survived the first dose, or 36.0 per cent 672 chicks survived the second dose. Ninety three and five tenths per cen of the chicks resistant to the first dose were resistant to the retest dose Chickens surviving 2 doses are nearly completely immune to following tests The evidence of this first generation brought out the fact that a selectio and/or immunizing process must be initiated by the first contact with th disease. In the first case the first test would have eliminated the geneticall and environmentally susceptible birds. In the second case active immunit to the tumor would have been generated by contact with the tumor materia of the first inoculation. The later data indicate that both genetic an immunizing factors cooperated in the observed results.

Strains for further breeding have been selected toward resistance an toward susceptibility. Selection for resistance or susceptibility has bee by family tests. Progeny from families having the highest per cent surviva were selected as breeders for the resistant strains. Those showing zer survival were chosen for the susceptible strains. For the resistant strain breeding was from the surviving chickens. For the susceptible strain families were selected that had nearly zero survivors on the first test. Addi tional chickens were raised from these families. Matings within this group of cockerels and pullets formed the second generation of the susceptible strains. Selection for susceptibility consequently falls behind that for resistance. Unselected commercial chicks formed the control groups.

Selection for resistance has been maintained for 6 generations. At face value the trend of survival in the 6 generations is peculiar. Successive groups of chicks were tested within each of the early generations. The groups from the parental tests were rather uniform, 37.3, 38.0, 46.7, and 31.7 per cents surviving the four tests. The first three tests of the next, or first selected generation, progeny were likewise uniform; 75, 63, and 75 per cent survived. It would seem that substantial progress in selection for genetic resistance was in progress. That hypothesis, however, was suddenly contradicted by the survival values of the fourth and fifth lots of chicks from this first selected generation. These tests uniformly showed less than one half the survival of the 3 preceding groups of chickens, 33 and 36 per cent. On the basis of the tests these chicks had no better survival than their parental generation. The second generation of selected progeny from the survivors of the first generation likewise continued to show uniformly lower survival rates than those of the first generation, 20 per cent. Fourth generation selections were low and comparable with the third generation, 20 per cent. The fifth generation showed a rise in resistance, 27 per cent. The sixth generation chicks selected for resistance showed a further rise in resistance, 40 per cent. This is a peculiar selection curve but, as shown later, there may be sound reasons that account for its form. For diseases caused by pathogens, selection is dealing not only with the genotypes of the hosts, but also with genotypes of the pathogen.

When selection for susceptibility is practiced it is necessary to disease-test groups of family progeny before the families for further breeding may be selected. The selected parents must then raise second flocks as parents for the following generation. In consequence, the selections for the susceptible strains are constantly falling behind the selections for resistant strains in generation time and number. The first group of selected susceptible chicks had a survival rate of 49 per cent as against 75 per cent for the resistant group, but the following 2 groups averaged but 21 per cent survival, compared with 34 per cent for the resistant selections. As with the resistant selections of this period, the break in survival was sharp. Since selection for susceptibility was being practiced, it might be argued that progress in molding the host genotypes by selection was being attained. However, that conclusion need not be true. As distinguished from results obtained in resistant birds, it is conceivable that a mutative change in the tumor virulence may have been selected out as the tumor is passed successively through the control chickens, so that the pathogen is more virulent cell for cell, and more able to establish a tumor in the host than the tumor material with which the research started.

The breeding selections for susceptible birds had to be discontinued for 2 generations due to lack of space; it was likewise necessary to discontinue selection of birds for test for this reason and because of the necessity of expanding the stock. Testing was resumed for the fourth- and fifth-gen eration birds. The survival values for these generations were similar, 30 an 25 per cent. Compared with selection for the resistant strains, the result favor some progress toward susceptible genotypes. The susceptible selec tions reemphasized the question brought out in the resistant selection trends Why did survival decrease sharply at the same period in the selections Was it due to hosts, environment, or the tumor inoculation?

The Tumor Factor in Leukosis

The problem of leukosis tumor virulence may be compared with othe forms of pathogens in disease. We have shown that sudden changes i pathogen virulence occur in bacterial disease, *Salmonella typhimuriu* (Gowen, 1945; Zelle, 1942), rarely, and in *Salmonella gallinarum* more fre quently (Gowen, 1945, 1937–1954). The viruses of tobacco mosaic ma change in virulence with equal suddenness. These changes meet the criteri of mutative changes. The method of change in the leukosis tumor virulenc may be postulated as follows.

In practice, the tumor is transferred to a new chick every 15 to 20 days Samples of cells from the previous passage tumor were inoculated into chick to obtain the next passage tumor. At each passage the cells were subjecte to such selection pressures that those cells most capable of rapid growth were favored to establish the new tumor. The numbers of inoculated cells are large. Changes in virulence may occur in any cell at any passage, but evidently do not occur frequently. However, when they do occur the selectio is for any cell that has a high growth rate and ability to establish rapidly i the chick. The necessary testing of our selected chicks in subgroups wa ideal for observing such changes. In each generation the chicks were al random samples of the same parents, so that the different host groups of any one generation did not differ appreciably in resistance. The sharp chang in virulence between the third and fourth tests was indicative of an intervening mutation to higher virulence in some tumor cell, with the replacement of the tumor line by the cell progeny of this cell. The experimenta arrangements were such that the tumor of the higher virulence shoul retain its virulence. Our subsequent tests over about 85 passages hav indicated that it does.

This hypothesis was tested further. Another sample of the frozen RPI 12 tumor was obtained. Cells of this tumor were passed twice throug control chickens. The resulting cells were used as the third passage cells o third-generation resistant, first-generation susceptible, and control chicks Tumor of the now virulent type 41–47 passage also was used as inoculum o corresponding chicks of the same generations. Three criteria were used t measure the effects: percentage of survival, days survived of the birds tha did die, and size of organs significant in this disease.

By each criterion the 41–47 passage tumor, when tested at the same tim on comparable chicks, was more virulent than the third-passage tumo although each traced back to the same group of cells. The pooled result of these tests show that the rate of chick survival for the 41–47 passage tumors was 6.5 per cent, while that for the chicks inoculated with the 3–4 passage tumor was 47.4 per cent. Mean numbers of days for the chicks that died from inoculation to death were 13.6 for the 41–47 passage tumor and 28.7 for the 3–4 passage tumor. The 41–47 passage tumor inoculum brought about a generalized reaction in 84.6 per cent of the chickens, while the 3–4 passage tumor inoculum caused only 19.8 per cent of its cases to become generalized. With the reduced virulence the reaction was generally localized in the breast muscle, the tumor became huge, and death appeared to result from its immediate effects. In the case of the virulent inoculum the breast muscle tumor was much smaller, internal organs became involved, and death seemed to result from the more generalized disease. The earlier deaths from the virulent inoculum, as contrasted with the avirulent, clearly influenced the clinical expression of the two tumors. In quantitative terms, breast muscles of the chicks inoculated with the virulent tumor averaged 7.1 gm., while those for the chicks inoculated with the avirulent tumor averaged 27.1 gm., the normal controls being 3.4 gm. Avirulent tumors often exceeded the virulent tumors in weight by 5 to 9 inches. On the other hand, the avirulent tumors seldom metastasized to other organs, whereas the virulent did so. Livers of birds inoculated with the virulent tumor line weighed 15.4 gm., whereas those for the avirulent tumor line birds weighed 8.4 gm., both on the average. Since these comparisons were made, there have been some 60 passages of the tumor cells of the 2-4 passage tumor through living hosts. The cells of these latter passages are now as virulent as those derived from the first tumor.

The relative liabilities of different host selections to inoculated tumor cells have been investigated by comparing the susceptibilities of chicks from mating within and among resistant and susceptible strains. Six such quadruple comparisons have been made. These tests were made with tumor inoculum diluted 1 to 640 saline, as dilution experiments have shown that this dilution brings out greater differences in the hosts. These tests show that the strains do differ in the direction that the selections would indicate. In general, the resistant selections are noticeably more resistant than the susceptible. Differences between reciprocal crosses are not large. The hybrids between the resistant strains and susceptible strains tended to resemble the resistant parents, indicating phenotypic dominance of resistance. Differentiation of the strains seemed to be taking place under the pressure of the selection that was exercised. It is premature to define the exact nature of the inheritance involved. As the results stand, they resemble those of our previous work on mice in which the inheritance depends on several gene loci rather than on a single locus.

In Vitro Immune Factors in Leukosis

The mechanism through which the genes act to bring about resistance or susceptibility is an important phase of the genetic problem. Working with Haensly (1956), we attacked this problem from two directions. It will be recalled that birds surviving one inoculation of the tumor are nearly all resistant. When the chicks have withstood two inoculations they ca be considered nearly immune. The factors that led to this immunity i chicks treated in this manner were sought in experiments approaching th problem from two different directions.

The birds could be immune because of circulating antigens, lysogenic o neutralizing bodies that might be found in their blood or organs or, in high concentration, in some tissues that may form them. To analyze for thi possibility, one hundred and forty cockerels immune to 9 successive inoculations of the leukosis tumor cells were chosen to furnish the sera and organs used in the search for organ foci of the observed immunity. The work was directed toward tracing and separating the sources of the substances involve in this immunity. Different organs were isolated from the immune an control birds. The following organs were studied: blood, duodenum, heart liver, lungs, pancreas, proventriculus, spleen, testis, bone marrow, brain cloaca, crop, esophagus, gizzard, large intestine, muscle, small intestine, an kidney. The organs were homogenized separately and incubated wit similarly homogenized tumor tissue for varying lengths of time. In this manner any immune substances present in the tissue had an opportunity to act on the tumor cells and exert any immunizing effect. The mixture was then introduced into the left pectoral muscle of fresh lots of test chicks. After a suitable time interval for tumors to develop and cause death, the remaining surviving chicks were reinoculated in the right pectoral muscle with untreated virulent tumor in large doses to test for either active or passive immunity of the surviving birds.

The tests were made in 3 ways. Organ tissue and tumor tissue were mixed in 1:1 and 1:½ proportions immediately following removal, allowed to incubate, and then inoculated into test chicks. In the third method, organ tissue was injected into the left pectoral muscle of the chick 2 days prior to inoculation of the tumor tissue.

The results of these extensive tests for the mechanism of resistance to the tumors revealed little more than the fact that the mechanism was not simple. Customary humoral antibodies, if present, did not affect resistance. There were differences in the effectiveness of different organs, but the organs of the controls, in general, did as well as those from the immune cockerels. Prior treatment with organ cells or serum 2 days in advance of tumor injection, if anything, favored tumor "takes" and growth. The pancreas tissue from either the immune or control birds showed an inhibitory action on the tumor tissue, whereas the other 19 organs did not. This may be due to the digestive enzymes secreted by this organ rather than its direct immunological effect. Other cells causing a reduction of tumor takes were derived from the esophagus, gizzard, cloaca, heart, liver, lungs, proventriculus, small intestine, spleen, and testis. Cells from immune organs, the small intestine, brain, crop, liver, spleen, testis, cloaca, esophagus, and heart stimulated resistance to challenge tumor tests. Despite the volume of this research there appeared no clean-cut separation of causative agents for tumor resistance. A new approach to the problem was necessary.

In Vivo Factors in Leukosis

The second approach allows the living breast muscle cells to work on the tumor cells over various lengths of time. Four experiments, involving 44 paired immune and control birds, have been conducted. Tumor cells were inoculated into the right pectoral muscle in either 1-gm. or 5-gm. amounts. In the first experiment the birds' cells were allowed to act on the transplant tumor cells and possible contained virus for varying lengths of time: 2 hours, 2, 4, 8, 16, 32, 64 days. The first experiment showed that the critical time for action on the tumor was between 0 and 8 days. The 3 subsequent experiments were held within that range. Four treatments are of interest: right pectoral muscle of resistant birds plus tumor cells; left pectoral muscle of resistant birds without inoculated tumor cells; right pectoral muscle of control chickens plus tumor cells; and left pectoral muscle of control birds without inoculated tumor cells. After the tumor cells were exposed to the pectoral muscles, the muscles plus tumor or muscles were dissected out of the birds, homogenized, and assayed for tumor-producing capabilities by inoculation into chicks. In assaying, the muscle plus inoculated tumor cells was tested in 4 dilutions with saline, 1:2, 1:5, 1:20, and 1:80. There was a minimum of 5 chicks and a maximum of 40 chicks for each of the 80 points representing a specific test, time, and dilution.

The results indicated that association of the tumor cells with the immune cells for somewhat more than one day was necessary to destroy the tumor-producing characteristics. The second point of interest was the appearance of tumors in the 1:5 dilution on the eighth day. Apparently some of the tumor cells escaped the action of the immune cells during that period.

No tumors resulted from any of the inoculations of the left pectoral muscle material that was not previously treated with tumor cells. This material came from birds that had had saline inoculated into their left pectoral muscles when tumor cells were injected into their right pectoral muscles. The contrasting results for the breast muscles on the right and left sides of the body indicated that no transfer of tumor substances or cells took place between the pectoral muscles on the two sides of the chicken within the 8-day period.

Tumor cells inoculated into the right pectoral muscle of the control birds persisted in the muscle for 2 days. The tumor cells were not inhibited in their growth. By the fourth day the inoculated tumor cells had increased noticeably in numbers, causing most of the samples from the 1:2 and 1:5 dilutions to show tumors when inoculated into test chicks. By the eighth day nearly all of the right breast muscle tissues contained tumor cells.

The contrast of this result with that for the immune birds was striking and enlightening. The immune pectoral cells certainly had the capacity to destroy tumor cells when integrated with the rest of the functioning animal. This was in contrast to what was observed when we compared *in vitro* action of the pectoral muscles on the tumor cells as described earlier. Two reasons for the differences were evident. The *in vitro* treatment was of short duration. A longer time may be necessary for the immune substances to act o the tumor cells and/or their contained virus. Second, in these experiment the pectoral muscle cells were fully functioning, whereas the *in vitro* cell probably were not, thus leading to greater effects on the tumor cells.

Inoculating saline-treated left pectoral muscle cells of the control bird into test chickens gave only one tumor out of the many tests. This showe again that the tumor cells or their virus did not often migrate to the othe side of the bird in the 8-day observation period. This observation was als true for further data up to 64 days from inoculation.

Discussion

The main objective of this research has been to establish strains of chicken consistently susceptible to the leukosis tumor RPL 12 to contrast with those consistently resistant to this tumor. The work is still in its preliminary stages. How far the strains are from the objectives sought may be judge from the report.

In this work there is some evidence of progress toward genetic differentiation of the strains toward resistance and susceptibility. The data indicate that multiple factors, rather than genes in a single primary locus, are involved (Asmundson and Biely, 1932). This result would seem to conform to the data of Hutt and Cole (1947, 1953) and their associates in their studie of the effects of selection in establishing strains of White Leghorns resistant or susceptible to natural leukosis infection, although the types of resistance sought were different, the gene bases for resistance may well be different (Heisdorf *et al.*, 1947).

Evidence is presented on the stability of tumor-cell virulence transferred through chick passage. Change in infectivity and lethality occurred twice during the experiments. Both changes occurred suddenly and were toward increased virulence. The changes came after two or more passages of tumor cells in living chicks following preservation in the frozen state. The changes in virulence were like those observed in our laboratory in *Salmonella typhimurium* and *Salmonella gallinarum* (Zelle, 1942; Gowen *et al.*, 1937–1954). The virulence changes resemble those induced by mutation. They are compatible with what may occur in other tumors, although some will point to the long record of stable virulence in many of the best-known laboratory transferred tumors. Similar variations in stability occur in the bacteria. In our hands, variations in virulence of our 11C strain of *S. typhimurium* are much less frequent than those that are found for our line of *S. gallinarum.*

Our studies of the causes for the resistance to leukosis virus found in birds that have survived 2 or more inoculations of virulent tumor favor long-time effects rather than rapidly neutralizing antibodies. Treatment of tumo tissue *in vitro* with cells or sera from blood and from about 18 organs did not give clear-cut reductions in virulence of the tumor cells.

Studies of tumor tissue exposed to living breast muscle cells did give evidence for the destruction or reduction of these tumor cells to avirulence A lapse of some days was necessary before the tumor cells became inactive These results suggest that the immune muscle cells, rather than circulating umoral elements, were the direct agents controlling the tumor cells. This onclusion conforms to that reached in some of our recent studies (Stadler, 956; Stadler and Gowen, 1957a, 1957b, 1957c) on *Salmonella typhimurium.*

Summary

This paper presents some of our results on the effects of the genotype of he chicken on resistance to leukosis tumor inoculations. The results are till those of the early stages of a time-consuming experiment. Some progress was made toward selecting for genetic resistance or susceptibility to leukosis tumor cells. The differences did not appear to be humoral in nature in birds made resistant to leukosis tumors by their genotype in conjunction with two or more previous inoculations of leukosis tumor cells. The significance of the direct action of living body cells was brought out by the action of immune as contrasted with unselected breast muscle cells on tumor cells over an eight-day period. The immune cells destroyed the infectiousness of the tumor cells, whereas the unselected breast muscle cells did not. The results attained were the product of the joint efforts of R. P. Poirier, Janice Stadler, Eugene C. Seu, and William E. Haensly, working in our laboratory.

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GENETICS OF HUMAN CANCER: A GENERAL SURVEY OF METHODS

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This article is not intended as a review of the literature on the genetics of human cancer, but as a general survey of the methods that have been used. The choice of examples cited is to some extent arbitrary.

While certain genes have been shown to modify the incidence of specific tumors in mice, none have been found with the dramatic effects of some human genes. Polyposis intestini almost invariably terminates as carcinoma of the colon unless the diseased bowel is resected. Similarly, xeroderma pigmentosum dooms the patient to skin cancer unless precautions are taken to shield him from light. Perhaps the most interesting of these rare disorders is retinoblastoma, the genetic basis of which has been reviewed by Weller (1941) and by Griffith and Sorsby (1944). The condition is mercifully very rare and is a disease of early childhood. The tumor may be present at birth, and a diagnosis made on a patient over the age of 10 should be regarded with suspicion. One of the most interesting features of the tumor is its tendency toward spontaneous regression, which is far greater than is the case with any other type of malignant tumor. Unfortunately, one cannot rely upon the occurrence of this tendency, and prompt treatment is essential. Following regression there is a very characteristic scar on the retina. To some extent the disease behaves as if determined by a single dominant gene with incomplete penetrance. Sometimes an apparently normal parent shows the characteristic scar on the retina, but this is not always the case, and it may be found that the patient has an affected uncle or aunt. In those cases where there is an affected father or where there is a history of the disease in a close relative of a parent, the proportion of affected children is close to 50 per cent. However, when the mother was affected, 13 out of 17 children were found to have the disease (76.5 per cent). The numbers are small and perhaps represent no more than biased reporting, and further data would be welcome. One must also consider the isolated cases that may occur in quite large families. It is unlikely that all of them represent genetic mutants, since it is usual for only one eye to be affected in sporadic cases, whereas both eyes are often affected in the familial cases. Occurrence of the disease might be due to somatic mutation in the retinal anlage. Hemmes (1931) and Lange (1938) consider that there is a tendency for sporadic cases of retinoblastoma to occur in the first-born. This finding is not really favorable to the hypothesis of somatic or germinal mutation. There can be little doubt that a liability to develop retinoblastoma is strongly conditioned by a single gene that must be continually renewed by genetic mutation. Perhaps this gene determines susceptibility to a virus, other genotypes succumbing very rarely to its action. Such a view is quite consistent with the high incidence of the disease in the children of affected mothers and in the tendency for sporadic cases to occur early in the family. A similar phenomenon is known to occur in mouse leukemia (MacDowe and Taylor, 1948).

Interesting though these rare genes are, they make a very small contribution to the total incidence of malignant disease. The only commo genes known to have any effects are the ABO genes. Even in this case th over-all effect is relatively small.

Since it is obvious that neoplastic diseases will not yield to any direc Mendelian interpretation, it is essential to make a different approach. I genetic constitution has a marked influence on the likelihood of contractin malignant disease, there should be an excessive concentration of such conditions in certain families. The general public and even certain scientists refe to cancer as though it were a single entity like syphilis or tuberculosis, which may happen to show different local manifestations. That this is incorrect i shown clearly by genetic studies in mice and by a perusal of human statistics This does not mean that there is no final common path such as somatic mutation leading to malignancy, but that the steps leading to this change are widely different in various manifestations of neoplastic disease. A further complication must be taken into account by the student of human genetics Diagnostic skills have changed a great deal in recent years, in spite of which diagnostic accuracy varies a great deal for different sites. Thus one coul seldom be sure of the validity of death certificates issued twenty years ag if one were investigating lung cancer; a similar uncertainty exists today i investigations of cancer of the pancreas. Useful inquiry is therefore limited to neoplasms at certain sites. Finally, once one has shown that some particular form of cancer shows concentration in certain families, there remains the question of interpretation. Families share a number of things other than genes, such as dietary habits and religious beliefs, with their accompanying sexual and social patterns. We must therefore look for ancillary evidence before any interpretations can be made. In human genetics we cannot follow the methods used in animals but, in their place, we have the study of twins.

Families with a very high incidence of certain forms of cancer have often been reported in the literature. It must be remembered that, if any common form of cancer, for example mammary cancer, is distributed at random in the population, some families will have several cases, and that such families will probably attract attention. Not all such families can be dismissed as chance occurrences. The well-known "G" family of Warthin is one such case. The most recent account of this family was given by Hauser and Weller (1936). The males had a very high incidence of carcinoma of the large intestine; the females had a very high incidence of carcinoma of the corpus uteri. This was probably a very rare gene with different effects in the two sexes and would be included more properly among the special cases mentioned above.

Clearly, if we wish to obtain information of general interest, investigations must be systematic. Much of the pertinent data has been reviewed elsewhere (Gorer, 1953) and here we shall deal only with the salient points.

In order to assess the results, two methods have been employed. One i to compare the figures obtained from the families of cancer patients with a control group, the other is to compare them with the deaths expected for the population as a whole. Sometimes both methods have been used in the same investigation.

Table 1 summarizes the results obtained by Videbaek and Mosbech (1954) in an investigation of 302 families of patients with carcinoma of the stomach. Of the controls, 55 per cent were old age pensioners, 19 per cent were employees from two factories, 12 per cent were hospital employees, and 14 per cent were friends and acquaintances of the authors. The figures obtained from the proband and control series were also compared with the expectations based on the mortality in Denmark for the years 1931 to 1940.

Table 1
Family Studies in Cancer of the Stomach*

Relation	Patients	Control	Expected†
Fathers	36	8	19.5
Brothers	33	8	20.0
Paternal uncles	8	6	14.0
Maternal uncles	5	10	14.5
Sons	2	2	1.0
Males, total	84	34	68.4
Mothers	25	8	18.0
Sisters	22	4	16.0
Paternal aunts	9	7	10.0
Maternal aunts	8	5	15.0
Daughters	1	0	0.5
Females, total	65	24	59.8

* Abbreviated from Vidabaek and Mosbech (1954).

† On statistics for Denmark, 1931–1940.

It will be seen that the incidence of gastric cancer in the control series is considerably lower than the calculated incidence. It is not at all obvious why this should be so. It is extremely difficult to obtain really satisfactory control material. Ideally, controls should come from the same environment as the patients. In practice, such controls are very difficult to obtain. Relatives of cancer patients are often willing to cooperate, while those with trivial complaints seldom will do so. In England the incidence of tumors from the lip to the pylorus appears to rise steeply with increasing poverty (*Registrar General*, 1938). The Danish controls are socially heterogeneous, but they do not show any bias toward the more prosperous classes. It must be remembered that, if carcinoma of the stomach shows an undue concentration in certain families, one should find other families in which the disease should be rare. Even if one has misgivings concerning the validity of the Danish controls, the results are still impressive. Among the fathers and brothers of probands there were 69 cases of carcinoma of the stomach against an expectation of 39.5, while for mothers and sisters the figures are 47 and 34 respectively.

The question of carcinoma of the breast has been reviewed elsewhere (Gorer, 1953). A number of completed investigations vary considerably in merit. All save one agree that carcinoma mammae does show a concentration in certain families. The dissentient vote comes from Passey and Wainman (1942), who failed to find any excess of mammary cancer in the mothers and sisters of patients. The account given is very brief. Therefore one cannot see why their result should be so different from those of Penrose *et al.* (1948), who found 25 cases of mammary cancer in the mothers of patients with an expectancy of 11 cases, while with sisters the figures were 23 and 7 respectively.

As might be expected, investigations that have dealt with cancer in general have given conflicting results.

There are a number of very striking cases of almost simultaneous occurrences of similar tumors in monozygotic twins to be found in the literature. The probability that two such people will develop the same type of tumor simultaneously is very small, even if the tumor is of a common type. These dramatic cases cannot be dismissed as purely fortuitous; they probably indicate that the affected twins were of a highly susceptible genotype. Unfortunately, they do not tell us how common these types may be. To discover this it is necessary to study twins systematically. The largest of such investigations have been made by von Verschuer and Kober (1940) in Germany and by Busk *et al.* (1948) in Denmark. In both series a large number of cancer patients were questioned concerning their status as members of pairs of twins. If the answer was affirmative, further inquiries were made to see if the pair was monozygotic or dizygotic. A total of 47,000 cancer patients was investigated by these two teams; from the total, 262 "useful" pairs were obtained. The number of multiple pregnancies in Western Europe is more than 1 per cent, and the proportion of useful pairs may therefore seem unduly low. However, neonatal mortality is considerably higher in twins than in single births. Furthermore, there are cases in which the fate of a member of a pair could not be ascertained, and it sometimes happens that when one twin has died in infancy the surviving member may be ignorant of the fact that he is a twin.

The results of these two great investigations are shown in TABLE 2. It will be seen that Busk *et al.* (1948) give the degree of concordance to be expected by chance for a population of the sex and age distribution under consideration. Clearly, there is no indication that highly susceptible genotypes are very common in Denmark or Germany.

Before analyzing these data further we may point out that there are two main variables to be considered in the present context: the time at which a tumor is observed, and its situation. The figures sited above indicate that the time of onset of neoplastic disease is not very strictly determined genetically. This should cause no surprise to students of mouse genetics. However, TABLE 3 shows that the site at which a tumor is likely to occur is largely

TABLE 2
CANCER IN TWINS: LARGE UNSELECTED SERIES ONLY*

Type of twin	Concordant observed	Concordant expected	Dis-cordant	Total	Con-cordant	Dis-cordant	Total
Monozygotic....	7	5.0	43	50	2 (4)	21 (19)	23
Dizygotic.......	7	9.2	126	133	7 (9)	49 (47)	56

Figures in parentheses are those found by Kober in 1950. Quoted by von Verschuer (1953).

* Data from Busk *et al.* (1948) and from von Verschuer and Kober (1940).

TABLE 3
DEGREE OF CONCORDANCE FOR TYPE OF GROWTH WHEN BOTH MEMBERS OF A PAIR ARE AFFECTED: UNSELECTED SERIES ONLY

	Concordant	Discordant*	Total
Monozygotic..........	12	4	16
Dizygotic.............	5	18	23
Total..............	17	22	39

* In two of these, one partner had carcinoma of the stomach, the other of the intestine (see text).

under genetic control. If both members of a monozygotic pair do develop tumors, it will probably be in the same organ. In two of the four discordant monozygotic pairs, one twin had carcinoma of the stomach, and the other had carcinoma of the large intestine. Among dizygotic pairs the agreement is less striking, but some of these pairs were of different sex.

The data presented in TABLE 2 suffer from the grave drawback that they treat cancer as if it were measles or tuberculosis. It may seem superfluous to expose this fallacy further, but its importance is well shown in reaching a decision concerning the inclusion of any twin pair in the series under discussion. In any systematic survey one will find partners who are alive and healthy, those who have developed malignant disease prior to the survey, those who have done so during the survey, and those who have died prior to the occurrence of malignant disease in the proband twin. At what age should the death of a partner disqualify a twin pair from inclusion? The Danish workers excluded twin partners who had died prior to the age of five. If the proband had retinoblastoma or acute leukemia, this might be legitimate, but it is hardly suitable if the proband has gastric or mammary carcinoma.

The only two sites that warrant detailed discussion are carcinoma of the stomach and carcinoma mammae. Before considering these, it will be as well to consider certain theoretical points affecting questions of interpretation. We may draw a comparison between the results of twin studies and the results that might be obtained if one were to select pairs of mice from various inbred strains. Clearly, the number of concordant pairs must depend upon the relative frequency of susceptible and resistant strains. However, even if we draw pairs from a single susceptible strain, the degree of concordance will depend upon the time during which the observations are made. In TABLE 4 we see the data of Bittner (1935) for the A strain of mice.

TABLE 4
CANCER INCIDENCE IN A HIGHLY SUSCEPTIBLE STRAIN OF MICE*

Age (months)	4.5–5.5	6.5–7.5	8.5–9.5	10.5–11.5	12.5–13.5	14.5–15.5	16.5 and over
No. dying							
Cancer-free	35	32	26	23	17	15	4
With cancer	3	20	49	62	64	37	34

* Data from Bittner (1935).

TABLE 5
CANCER OF THE STOMACH IN TWINS: UNSELECTED MATERIAL ONLY

Authors	Monozygotic pairs			
	No. of pairs	Discordant	Same tumor	Different tumor
von Verschuer and Kober	6	4 (2)	2 (4)	—
Busk and associates	6	5	—	1*
Others†	7	3	3	1*
Total	19	12 (10)	5 (7)	2*

Authors	Dizygotic pairs			
	No. of pairs	Discordant	Same tumor	Different tumor
von Verschuer and Kober	17	13 (12)	— (1)	4
Busk and associates	8	6	—	1
Others	9	4	1	4‡
Total	34	23 (22)	1 (2)	9

The figures in parentheses include the more recent data of Kober, as in TABLE 2.
* These tumors were both in the large intestine.
† References from Gorer (1953).
‡ One with tumor of large intestine.

No tumor was seen earlier than 4.5 months of age, and deaths prior to that age do not enter the calculations. Subsequently 64 per cent of 421 mice developed mammary cancer. If we were to draw pairs at random from this population we should obtain some pairs in which neither had cancer. In twin investigations such pairs would not occur, since one member must have cancer to be included in the study. If these pairs are ignored, 47 per cent of pairs will be found to be concordant. However, this figure would be applicable only to human material if the latter were to be based on death certificates of human twins. In practice this is not true. The German survey was originally carried out between 1933 and 1938. A follow-up was done by Kober after 10 years (quoted by von Verschuer, 1953) and such details as are available will be dealt with later. Among the Danish series very few pairs had been under observation more than five years. If we wished to carry our analogy with mice further, we should have to consider what might happen if we were to observe the mice for a few months. It is futile to go into detailed calculations, but a glance at TABLE 4 will show that one half of the probands would be aged 11.5 months or less, and that many would have healthy partners, of which a large number would develop cancer later. One must therefore be cautious in drawing negative evidence.

TABLE 6
MAMMARY CANCER IN TWINS: UNSELECTED SERIES ONLY

Authors	Monozygotic pairs		
	No. of pairs	Discordant	Concordant
von Verschuer and Kober	3	3	—
Busk and associates	12	11	1
Others*	2	2	—
Total	17	16	1

Authors	Dizygotic pairs		
	No. of pairs	Discordant	Concordant
von Verschuer and Kober	9	8 (7)	1 (2)
Busk and associates	19	17	2
Others*	8	5	3
Total	36	30 (29)	6† (7)

Figures in parentheses as in TABLES 2 and 5.
* References from Gorer (1953).
† In only one of these cases did both partners have cancer of the breast.

The data for cancer of the stomach given in TABLE 5 are very striking. The figures in parentheses show the results of Kober's follow-up. No less than 9 of 19 pairs show concordance if we include the smaller systematic surveys, or 5 of 12 if we confine our attention to the largest studies. This is an interesting result, but it should not be unexpected. Carcinoma of the stomach is certainly favored by certain types of environment, but its association with blood group A and with pernicious anemia and achlorhydria indicate that not all genotypes are equally susceptible. The data give strong confirmation to the findings of Videbaek and Mosbech.

In TABLE 6 we see the results for mammary cancer. Here there is but little indication that susceptible genotypes are common. Von Verschuer and Kober list only three pairs of monozygotic twins, and the age at notification is not stated. It will be interesting to see if a long-term follow-up of the Danish series alters the figures materially. At present the data resemble Passey and Wainman's findings.

To many the facts presented may seem somewhat disappointing, particularly with regard to studies of twins. In my opinion such studies are of great importance. They need to be carried out on an international scale; they also demand considerable patience. The establishment of pure strains of mice by Leonell C. Strong and others also needed considerable patience, which has been well rewarded. Considerable interest is now shown in the geographical distribution of various types of cancer. A knowledge of genetic variables is of vital importance in understanding the epidemiology of neoplastic diseases.

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STUDIES ON HUMAN CANCER FAMILIES

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The heterogeneity of human populations and a lack of confirming evidence both complicate genetic studies of human cancer. A family becomes part of a study because one of the members develops cancer. The investigator knows himself to be treading on unsound ground when he tries to follow the history back into earlier generations. Many records will have been lost. The memory of most of us about the illnesses of our ancestors is far from accurate.

Family histories of the rarer forms of cancer, however, can be studied genetically without too much difficulty. The disease occurs in only a few families in any population unit. Information about affected and non-affected members can be collected with relative ease. The patterns of inheritance have been determined for cancerous and precancerous types such as retinoblastoma, neurofibromatosis, multiple polyposis, and xeroderma pigmentosum.

Several reports have been made of families having multiple cases of more common types of cancer. Such histories are striking. The histories may include members having several different kinds of cancer or relatives with cancer of one site. A high frequency of cancer in a family group may be the result of gene action, but that is not necessarily the explanation. An environmental agent common to the relatives could theoretically cause multiple cases in a family. Moreover, cancer occurs so frequently in our population that mere chance might be the explanation for some cancerous families.

One might suppose, then, that family histories are of little value for genetic studies of common forms of cancer. That opinion should not be held too strongly. If geneticists fail to collect and complete family histories of cancer when the opportunity arises, they will miss an opportunity to determine patterns of inheritance once heredity has been shown to be a factor. There is a possibility, also, that cancers of a particular site and type, such as breast cancer, may be broken down into distinct entities by detailed studies of family histories.

Twin Studies

Some measure of the relative effects of heredity and environment as causes of cancer must be available for an understanding of the genetics of the disease. Monozygotic and dizygotic twins have been used for that purpose. If heredity is the only causal factor, monozygotic twin members will always be similar; dizygotic twin pairs will sometimes show similarity and sometimes unlikeness, just as do regular siblings. If environment is the sole causal agent, the frequency with which similar pairs occur should be approximately the same for the two kinds of twins. Relative effects of both heredity and environment are measured by comparisons of the proportions of similar, or concordant, and dissimilar, or discordant, pairs of monozygotic and dizygotic twin pairs.

Macklin (1940, 1947) reported the degrees of concordance for cancer among monozygotic and dizygotic twin pairs. The concordance for cancer occurrence was higher for 65 monozygotic than for 49 dizygotic twins by about two to one. However the proportion of discordant monozygotic twins was rather high, approximately 37 per cent, indicating that environment is a strong agent. Comparisons of twins with both members cancerous showed that monozygotic twins have a much higher degree of concordance for similar sites than do dizygotic twins.

Busk, Clemmesen, and Nielsen (1948) collected information on cancer occurrence among 185 twin pairs in Denmark. They reported a slightly higher concordance among monozygotic than among dizygotic pairs for cancer occurrence, but the difference could not be considered significant when the expected proportions were determined after corrections for age. In only 7 of 52 monozygotic pairs and 7 of 133 dizygotic pairs were both members affected. Among these concordant pairs, the monozygotic twins showed a clear tendency for similar site involvement: 4 of the 7 monozygotic, in contrast to 1 of 7 dizygotic pairs.

Relatives of cancer probands might seem a good source for twins. Oliver and Jackson (1954) reported 548 twin pairs among relatives of probands used in their studies of breast cancer. The results show the difficulties encountered in collecting twins for study. Only 91 of the pairs could be used. The others were discarded because they were younger than 30 years, or because one or both had died in early life, or because information was in some way scanty. Verifications of the reported type of twinning have not been made for all pairs. According to the information available, however, 6 of 23 monozygotic pairs and 22 of 68 dizygotic pairs have one or both members affected. Three of the 6 monozygotic and 3 of the 22 dizygotic pairs show concordance for cancer occurrence. The pairs of twins are to be followed.

A number of reports have been made of single pairs of twins with both members affected. No attempt will be made here to review these reports. Monozygotic twins, when both are affected, tend to have very similar pathology, as Oliver (1950) reported for the pairs of twins with breast disorders whom he identified as LP and DZ. We need more case reports of twins having only one member affected, as well as of those with concordant pairs.

Although dizygotic twins have a rather high proportion of pairs with both members affected, monozygotic twins show a definitely higher tendency to have similar sites involved when both members have cancer. The data support an interpretation that a hereditary susceptibility is responsible for the development of cancer in specific sites by some persons. The data are also evidence that a proportion of the cancers are caused by environmental agents, or that environment is responsible for variations in age of onset.

Observed and Expected Cancer Frequencies in Relatives

Actual cancer frequencies among relatives of probands have been compared to expected frequencies as a means of determining whether or not a hereditary tendency for cancer exists. Expected frequencies were determined by use of morbidity and mortality rates for cancer, assuming that relatives of cancer probands faced the general population risk. Some investigators chose instead to use a group of control (noncancerous) probands; they used the cancer frequency in their relatives as a measure of expected frequencies. Accurate information about cancer in older, deceased generations of families or in distant relatives of probands was not easy to obtain. As a consequence, comparisons drawn are primarily of the parents and siblings of probands.

Several cancer sites have been studied, using the control group as a measure of frequency. For practical reasons, the studies discussed here will be limited to some of the reports on breast and stomach cancer. More complete references can be found in the reports mentioned.

In their completed reports of breast cancer studies, Martynova (1937), Jacobsen (1946), and Woolf (1955) used a control group for comparative frequencies. Martynova reported that 2.65 per cent of female relatives of 201 breast cancer patients were similarly affected. Only 0.17 per cent of female relatives of a control group taken from dental clinic patients developed breast cancer. A higher proportion of mothers than of sisters of the probands had breast cancer. The cancer families also had a higher rate of total cancer than did the control families. Some question about the relative ages of the cancer and control probands has arisen.

Jacobsen compared the family histories of 200 breast cancer probands with those of 200 control probands selected from patients under medical care and from residents in an institution for old people. The frequencies of breast cancer among mothers (10.5 per cent) and among sisters (3.4 per cent) of breast cancer probands was higher than the respective frequencies in the control group. Jacobsen also reported a higher rate of total cancer among parents and siblings in the cancer group. He concluded that a hereditary tendency for total cancer exists, as well as a tendency for breast cancer occurrence. Busk (1948) has shown that the expected cancer rate among Jacobsen's control families is much lower than would be expected on the basis of the population risk. He supports Jacobsen's conclusion that there is a hereditary tendency for breast cancer.

Woolf selected a group of control probands from several sources, but attempted to match them with 200 breast cancer probands for age, socioeconomic status, and sex. The sequential analysis method was used to compare the frequencies of breast cancer and total cancer among relatives in the two family groups. Woolf concluded that breast cancer is hereditarily conditioned, but that families having breast cancer do not have an increased tendency for cancer at other sites. These conclusions agree with the results he obtained by comparing the observed breast and total cancer rates with the rates expected when computed by use of Utah vital statistics.

The former type of study mentioned above compares the frequencies in cancer families with expected rates computed from the statistics of the general population. If heredity is a causal factor, families of cancer probands should have a rate higher than that for a population of the same age, sex, and environmental patterns. On the other hand, if the cancer families face the same risk as the population, the two rates should not differ significantly. Smithers (1948), Penrose, MacKenzie, and Karn (1948), and Woolf (1955) have compared the rate of death from cancer in families of breast cancer probands with the deaths expected if risks are the same as those of the population.

Smithers questioned 459 breast cancer patients about cancer among their relatives. He found that a higher number of deaths from breast cancer were reported for the mothers and sisters of the probands than would be expected on the basis of population risk. The frequency of cancer at other sites was not increased in the cancer families.

Penrose, MacKenzie, and Karn investigated the death rate from cancer among relatives of 510 breast cancer probands. The expected number of deaths was determined by matching the verified deaths from cancer in the families by age and year of death with the mortality rates for England and Wales. The number of deaths from breast cancer in 25 mothers was about twice the expected number. Among sisters of probands, 23 had died from breast cancer, whereas 6.98 was the expected number. The numbers of deaths from general cancer in close male and female relatives of the probands was approximately equal to the number expected. The authors concluded that in the families studied, heredity is a causal factor for breast cancer, and that the inheritance is for a specific organ involvement, not for cancer in general. Woolf's conclusions were similar, as has been mentioned.

Hagy (1954), Woolf (1955), and Macklin (1955) have recently reported studies of stomach cancer cases. Woolf selected as probands 200 individuals having pathologically proved stomach cancer who had died. Of the 810 parents and siblings who had died since 1910, the death certificates showed that 5.4 per cent died from carcinoma of the stomach. The frequency is significantly higher than that expected on the basis of the population risk. Relatives of probands showed a rate of cancer at other sites no higher than that in the general population.

Hagy interviewed 106 patients who were proved cases of gastric cancer. The frequency of gastric cancer among the relatives of these patients was compared with the frequency in relatives of 60 control persons who were under medical care, but did not have cancer. Hagy found that 1.2 per cent of the relatives of gastric cancer probands were similarly affected, and 0.5 per cent of the relatives of control probands had had stomach cancer. The difference is not statistically significant.

Macklin (1955) reported an increased incidence of cancer of the stomach and intestines over the rate in the general population. Gastric cancer was found to be about four times more common in close relatives of gastric cancer probands than would be expected if they faced the population risk.

We cannot ignore the fact that the data obtained by some investigators have led them to refuse to accept heredity as a factor in the occurrence of the more common cancers. Even those who believe that heredity is a factor causing cancer to occur in a specific site do not always find rates that agree with those reported by other workers. The variations in frequencies found in the statistical studies are understandable.

Selection of comparable cancer and control probands is one difficulty that investigators try to meet. The probands should not differ markedly for any condition that probably has an effect on cancer occurrence. Age and sex distributions must be similar for the two groups. In breast cancer studies, marital status and childbearing histories need to be similar. Both sets of probands must belong to similar socioeconomic and, possibly, national groups. Clemmesen (1949) called attention to the possibility that a high rate of cancer among close relatives may be traceable to a common social and economic background rather than to inheritance.

Confirming of cancerous and healthy members of families is also a difficult but necessary task. In our studies, being made by Dudley Jackson, Sr., and myself, indigent and private patients with breast cancer were at first chosen for comparison with patients having breast papilloma, benign breast disorders, and a noncancerous control group. Most of the indigent patients were unable to give usable family histories. Many could not tell the interviewer where to find their close relatives. The indigent group has been of little value to the studies.

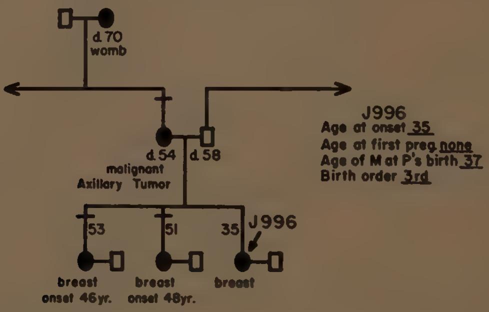


FIGURE 1. Breast cancer family J996. Cancer of mother was not so reported on death certificate.

Some persons, moreover, are reluctant to give information about cancer in their relatives. In some cases, individuals have feared that their families might suffer economically if it were revealed that they or their relatives were victims of cancer.

Occasionally office and hospital records are lost, making confirmation of cancers impossible. The death certificate does not always help; it may fail to show that a person had cancer before he died. In one of our cases, the mother of a breast cancer proband had had cancer, according to the doctor who operated (FIGURE 1). Her death certificate showed cause of death to be senility and thrombosis. It is of interest to know that the physician who signed the death certificate is the same one who reported to us that the woman had a cancer.

Breast cancer probands are not necessarily a common group, for more than one causal agent may be responsible for the development of cancers. This may explain the high frequency of cancer probands who have no relatives with breast cancer (TABLE 1). Among 312 breast cancer probands, we have found no evidence of cancerous relatives in 29.4 per cent of the families. The relatives include at least the parents' siblings. In another 38.3 per cent of the probands, no history of breast cancer in relatives has been discovered, although the families have cancer at other sites. Only 32.3 per cent of the probands have positive breast cancer family histories. Six per cent have relatives with breast cancer, but with no other types of cancer.

TABLE 1
PROPORTIONS OF BREAST CANCER AND CONTROL PROBANDS HAVING CANCER IN FAMILIES

Probands		Percentage of histories			
			Positive for		
Type	Number	Negative, no cancer	Breast only	Breast and others	Nonbreast only
Breast cancer....	312	29.4	6.1	26.2	38.3
Control.........	134	41.0	8.9	12.7	37.3

Control probands were taken from women who had consulted a physician for illness other than cancer. Among 134 of these probands, 41 per cent have no cancerous relative. Approximately 78 per cent have had no relative with breast cancer. Therefore, only 22 per cent have positive breast cancer histories. About 9 per cent of the histories are positive for breast cancer only.

Investigators using statistical methods for study of cancer rates have recognized the complications concerning control groups. They have attempted to meet the difficulties with extreme caution, knowing at the same time that someone will find fault with whatever control group is selected. The conclusions reached by most of the more recent investigators have been that mothers and sisters of breast cancer probands and the close relatives of stomach cancer probands face a greater hazard from cancer of the respective site than does the general population. The same conclusions have been reached when the observed rate has been compared with that expected on the basis of mortality rates.

Pedigree Studies

Although both the statistical method and twin studies have their place in our attempt to decipher the genetics of cancer, something more is needed. Pedigrees of cancer probands should be collected, and new data should be added as they become available. Possibly the cancerous members of a kinship will be found only in certain branches, just as some families have a high frequency of affected members, whereas other families have very few cancerous members.

Warthin (1925) began an investigation of a family having several members with gastrointestinal and uterine cancer. Hauser and Weller (1936) reported a further study of the family. Among 174 relatives 25 years of age or older, 41 had 43 cancers. Twenty-six, or 14.9 per cent, had cancer of the gastrointestinal tract and 15, or 8.6 per cent, had uterine cancer. The concentration of particular types of cancer may have been the reason the family was first reported. However, this remained a cancer family with the same concentration of types of cancer even after the follow-up study. Any bias that may have existed originally can be ignored.

Hagy (1955) studied the family of a woman who had gastric complaints but no cancer. Ten of her 135 relatives had cancer, and 6 of these had stomach cancer. The frequency of 4.4 per cent is not very high. If only relatives 40 or more years old are considered, however, 18.8 per cent had stomach cancer. Hagy discovered that actually the 6 with stomach cancer were the woman's paternal relatives. Only 19 of these relatives reached the age of 40, and the 6 with stomach cancer represent 31.6 per cent of the group. We find in this kinship that cancer of the stomach occurs in only one branch of the family. In contrast to these findings, Hagy (1954) found that only 1.2 per cent of the relatives of 86 stomach cancer probands were similarly affected.

Gardner and Stephens (1950) reported a kinship with 9 members having breast cancer and 4 having benign breast conditions. The frequency was considered high in comparison with Utah population rates. The concentration was limited to 3 branches of the kinship, representing 3 of 8 siblings and their descendants. Similar ages of onset and clinical histories were reported. A resurvey 3 years later (Gardner, 1954), showed 1 new case of breast cancer and 2 new cases of benign breast disorder.) These affected members belong to the same branches in which cases were first reported. The concentration involves not only the site of the disease, but also the particular branches of the kinship.

In another kinship (Woolf and Gardner, 1951), 8 cases of breast cancer have occurred in daughters and granddaughters of one woman who had developed breast cancer. Siblings of this woman have had as many descendants on the average as she herself has had, yet very few of these descendants have developed cancer.

The cancer families referred to represent only a portion of those reported in the literature. Although concentration within a family does not prove that heredity is the causal agent, the cancer families put the burden on us to explain why some families have so many members with the same site affected, and more particularly why only certain parts of the kinship have the affected members. The recording of a large number of histories of families having cancer would lend itself to investigations of the pattern of inheritance and possible environmental agents. Obviously, sporadic cases and families with only a small number of affected members cannot be ignored.

A pedigree will be of little genetic help unless the information about the relatives is complete and accurate. Follow-up studies should be made, also, to add new cases as they occur. Probands cannot be depended on to supply all the necessary information about their relatives, since they generally lack complete knowledge. Sometimes a proband reports a relative, even a sibling, to be cancerous although hospital records show otherwise. A report that a relative is free of cancer should be confirmed. A maternal aunt of a proband, in the studies by Jackson and Oliver, was reported as normal by several relatives. She herself gave us the correct information: she had had a mastectomy, and the pathological record confirmed the report of breast cancer. She had told none of her sisters or nieces about the disease. This same kinship shows why it is necessary to communicate with relatives of the probands. Case J134 had adenocarcinoma of the right breast. She knew about three relatives who had cancer; none had breast cancer. Other contacts revealed that seven members of the kinship have had breast cancer, and twelve have had cancers of other sites. A number of other members have had skin cancer and benign breast disorders.

Probands in our studies have been able to give more reliable information about their parents than about their siblings. The percentages of increased information gained after interviews with probands are given in TABLE 2.

TABLE 2
ADDITIONAL INFORMATION COLLECTED AFTER INTERVIEW WITH PROBAND

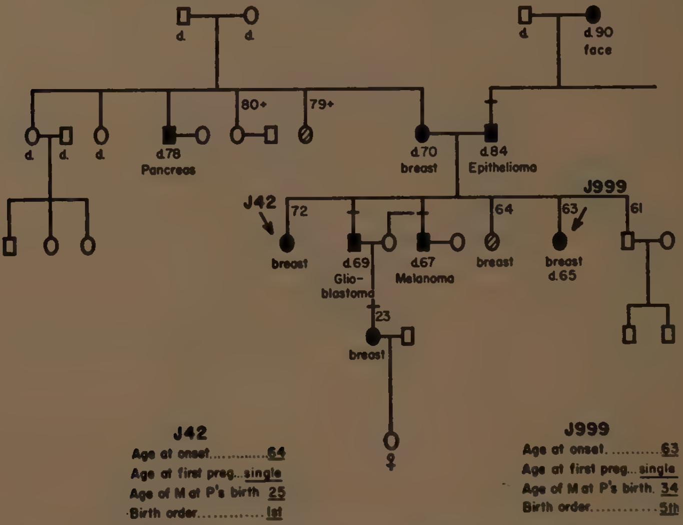
Probands' relatives	Cancer cases reported		
	By probands	Later	Percentage of increase
Mothers			
Breast	19	0	—
Other	30	4	13.3
Sisters			
Breast	23	5	22.0
Other	24	6	25.0
Brothers	24	11	46.0
Fathers	28	4	14.3

Nineteen mothers were reported by probands to have had breast cancer. Out checking with records and relatives has not changed that number. Probands reported that 30 of the mothers had had cancer at other sites. Four new cases were added, an increase of 13 per cent over the original number. Among sisters over 20 years of age, 5 breast cancer cases were added subsequent to interviews of probands, an increase of 22 per cent. For cancers at other sites the increase was 25 per cent. The increase for fathers was 14 per cent; for brothers, 46 per cent. As would be expected, the increase in information about more distant relatives was much higher:

126 per cent for relatives with breast cancer and 61 per cent for relatives with cancer at other sites.

Pedigree J42 has several cancerous members. The proband reported breast cancer in her mother and other cancers in her father, maternal uncle, and paternal grandmother. Subsequent cases in her relatives have increased the number of cancer cases to 9 (FIGURE 2).

Proband J899 in the Oliver and Jackson cases has had cancer of the left


d.
d.
d.
d. 90
face
80+
79+
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d.
d.
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Pancreas
d. 70
breast
d. 84
Epithelioma
J42
72
J999
64
63
61
breast
d. 69
Glio-
blastoma
d. 67
Melanoma
breast
breast
d. 65
23
breast
♀
J42
Age at onset............64
Age at first preg...single
Age of M at P's birth 25
Birth order............1st
J999
Age at onset...........63
Age at first preg...single
Age of M at P's birth. 34
Birth order............5th

FIGURE 2. Breast cancer family J42 in which siblings developed cancer subsequent to proband.

TABLE 3
THE J899 FAMILY RECORD*

Mother's siblings			Proband's generation	
Mother	d. 71		1. Dtr. d. 73 caB. 2. Dtr. d. 53 caU.v. 3. Son d. 69 4. Dtr. d. 63 caB.v. 5. Dtr. 65	6. Dtr. 63 vag. cyst 7. Prob. 60 caB.v. 8. Son d. 42 9. Dtr. 57 10. Son 52
Sister	d. 83		Dtr., dtr., son.	
Sister	d. 69	caS.v.	Dtr., son	
Brother	d. 80		Dtr.	
Brother	81		Son, son, dtr. bnB.v.	
Brother	78	caP.v.	Dtr., dtr bnU.v.	
Brother	d. 73	caL.v.	Dtr. bnB.v., dtr. bnU.v.	
Brother	d. 43	cab.	Dtr.	
Brother	d. 65		Son, son, son, son, dtr., caB.v. dtr.	

* Symbols: b = brain, bn = benign, B = breast, ca = cancer, d = dead, Dtr. = daughter, L = lung, P = prostate, S = stomach, U = uterus, v = verified.

breast and a basal cell cancer of the nose. Her kinship has several cancerous members. The information is shown in TABLE 3. Two of this proband's sisters had breast cancer; 1 other had cancer of the uterus. The woman's parents died free of cancer. Her mother had 2 sisters and 6 brothers, of whom 4 were cancerous (stomach, prostate, lung, and brain). Twenty children—12 daughters and 8 sons—were produced by the 8 siblings of the

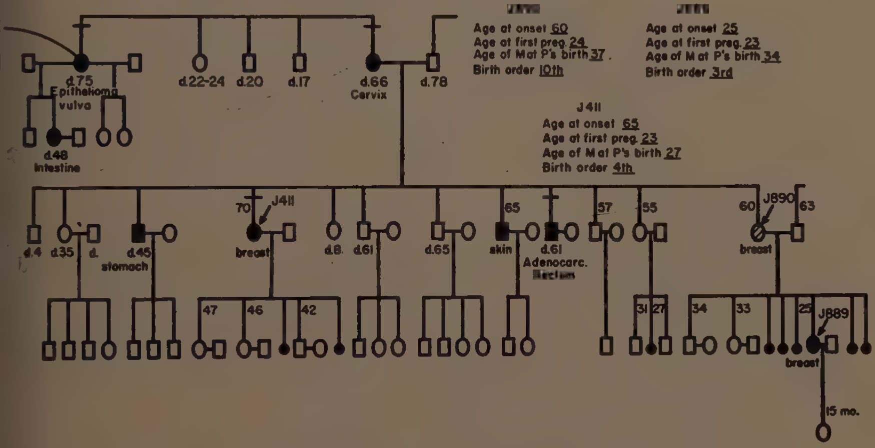

FIGURE 3. Cancer family J411 showing a variety of cancer types.

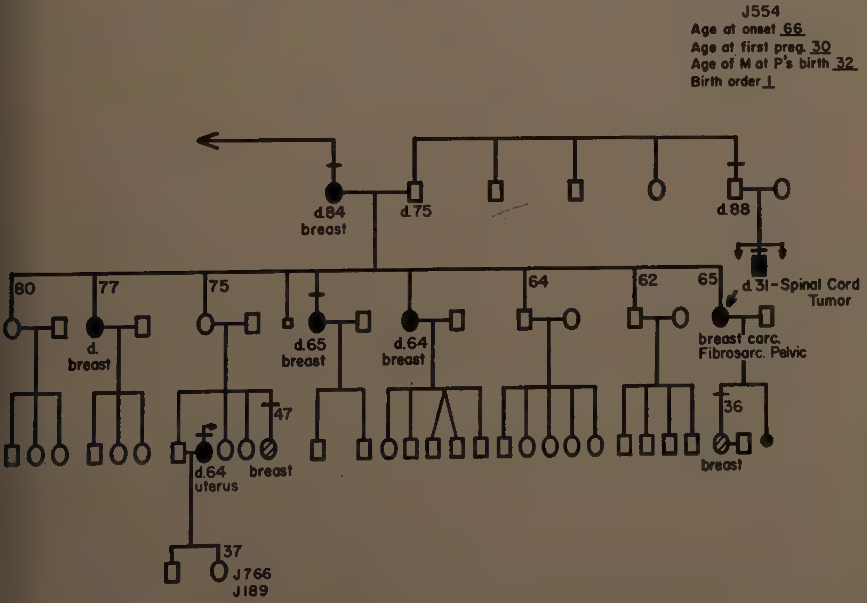

FIGURE 4. Breast cancer family J554. Information was gained from another kinship through a common relative.

mother. One of these daughters has had breast cancer, and 2 have had benign breast disorders.

Our filing system helps to add new cases in a kinship as they arise. Proband J411 had a record showing cancer of the cervix, stomach, and rectum in her siblings and mother. At a later time the proband's sister J890 came to the surgeon with her daughter, J889 (FIGURE 3). This sister had a benign breast disorder, and the daughter had carcinoma of the breast.

Sometimes one must enter by the back door, so to speak, to get information about a family. This was true for J554 (FIGURE 4). The proband's daughter refused to let her mother give any familial data to the interviewer nor would she allow us to go to her relatives. We did not contact the family, that being our practice when cooperation is refused. At a later time, however, J766 helped in the study. A member of this family who had uterine cancer had married a nephew of J554. A daughter of the couple helped the interviewer to obtain information about both her mother's and father's families. Surname cards connected J766 to J554. As a result, proband J554 is now reported as having a mother and three sisters with histories of breast cancer. The data were collected without breaking any confidence with the daughter of J554. The family was studied as relatives of the male who married into J766. No person was asked to discuss his or her family because of a relationship to J554.

Risks Faced by Relatives

One question invariably asked is whether a female relative of a breast cancer patient faces a greater-than-average risk of developing cancer at that site. The majority of the more recent investigators who have made statistical analyses of familial frequencies of breast cancer have concluded that mothers and sisters of the probands face an increased hazard.

Daughters of women with breast cancer are concerned about their risk. Information about the daughters of probands, after the generation has been completed, should give us the desired data. The probands will have been proven cancer cases, but the time needed for study will be long. Moreover, the probands in our studies whose average age of cancer onset was 53.2 years have been anything but fertile. Only 71 per cent of the probands have had any offspring. The average number per fertile proband is 2.6. There will not be many daughters for a follow-up. Cancerous aunts of the probands should have daughters of about the same age as the probands and their sisters, but confirmations of these relatives may be so incomplete that the data are not good.

Mothers of probands can be used for the study, although the number of these mothers with breast cancer will be small. Among 312 known mothers of our probands, 19 have had breast cancer; 34 have had cancer at other sites; and 259 have had no cancer, except possibly skin cancer. Some of the cancer-free group, about 20 per cent, died before the cancer age of their respective daughters. Possibly some of them would have developed breast cancer had they lived longer. They are nevertheless carried as noncancerous.

Breast cancer frequencies in sisters of probands whose mothers had breast cancer or no cancer are shown in TABLE 4. Mothers with cancer at other sites are omitted. The 19 probands with breast cancer mothers had 41 sisters who lived past the age of 20. Six of these sisters, or 14.6 per cent, have also had breast cancer. The 259 probands whose mothers remained free of cancer, disregarding age of death, had 576 sisters age 20 or over. Sixteen of these sisters, or 2.8 per cent, have had breast cancer. The difference is borderline with respect to significance; D/SE = 2.1. Under the circumstances, however, one is justified in concluding that daughters of mothers having breast cancer face a higher-than-average risk of developing a similar cancer. The 19 cancerous mothers were all of one type. The other mothers may have included some with a tendency for breast cancer who died at an early age and for that reason only are classified as cancer-free mothers.

TABLE 4
BREAST CANCER IN SISTERS OF PROBANDS HAVING MOTHERS WITH BREAST CANCER AND WITHOUT CANCER

Mothers of probands*		Sisters of probands†		
			Breast cancer	
Type	Number	Number	Number	Percentage
Breast cancer	19	41	6	14.6
No cancer	259	576	16	2.8
Totals	278	617	22	

* Omitted, 34 mothers with cancer at other sites.
† All sisters, 20 or more years old.

Mothers and sisters of breast cancer probands show in our cases a slightly higher rate of breast cancer than do the relatives of control probands. Controls were selected from patients under medical care having no history of tumors. Interviewers who worked with cancer probands also took records from 80 control probands. Another source of interviewers added 54 control probands. The 80 probands have 3 mothers and 2 sisters with breast cancer. The other 54 control probands have no mothers with breast cancer histories, but 2 of these probands' sisters have had breast cancer. The control probands include approximately the same proportion of childless women as do the cancer probands. Fertile control women produced an average of 2.55 progeny, and fertile cancer probands produced 2.6. Average age at interview for the control group was 50.0 years, for the cancer probands, 53.2 years.

Comparisons of cancer frequencies in mothers and sisters of the 2 groups are shown in TABLE 5. Among 312 mothers of breast cancer patients, 6.1 per cent have had cancers at that site. In contrast, 2.24 per cent of 134

Table 5
Cancer in Mothers of Breast Cancer and Control Probands

Probands		Cancerous mother of probands			
		Breast cancer		Other sites	
Type	Number	Number	Percentage	Number	Percentage
Cancer..........	312	19	6.10	34	16.0
Control.........	134	3	2.24	8	6.0

mothers of control probands have had breast cancer. The difference is just significant. If only the control group of 80 were considered, the difference between that group and the cancer proband group would not be significant. The observed frequency of breast cancer among mothers of breast cancer probands, 6.1 per cent, is not high. That is not so surprising if one remembers that only approximately 1 in 3 of the cancer probands have had any breast cancer relatives at all. This fact would not in any way vary the relative frequencies for the mothers of the 2 sets of probands. The observed frequency of breast cancer among mothers of the cancer probands is lower than that shown by the work by Jacobsen, higher than that by Woolf, and approximately the same as that by Penrose *et al.*, and by Smithers. The frequency in the control mother group is approximately the same as that shown by Woolf.

Breast cancer probands have had 696 sisters who lived to be 20 years of age or older. Among these sisters, 4 per cent have had breast cancer. In the control group, 1.5 per cent of 261 sisters have had breast cancer. Information about the sisters in the control group will be checked further before any definite conclusion is drawn. As the data now stand, the frequency in sisters of cancer probands is significantly higher than the frequency in the control group.

Summary

Studies of twins give strong evidence that cancer has an hereditary basis, but that environment is also a factor that must be considered. Particularly when both of a pair of twins are affected, monozygotic twins have the same site of cancer with a higher degree of concordance than do dizygotic twins.

Statistical studies have shown that parents and siblings of both stomach and breast cancer probands have a higher rate of cancer in the same site as that of the affected proband than would be expected if the families faced the general population risk. Those same relatives have cancers at other sites no more frequently than does the average comparable population. The data support the conclusion that in some families there is an hereditary basis for the occurrence of cancer at specific organs.

The study of pedigrees is essential for determining the pattern of inheritance. High concentrations of stomach or of breast cancer in families and in branches of families is evidence for inheritance of susceptibility to cancer of particular sites. Pedigrees can be used in the genetic studies only if confirmations are secured for relatives of the probands who are reported as healthy as well as those reported as cancerous. Cancer frequency is increased considerably above that reported by probands if relatives are contacted. This is true for siblings more than for parents of probands, but it is especially so for more distant relatives.

Not all probands have breast cancer members among their relatives. In 68 per cent of our records the proband is the only breast cancer case in her kinship, including at least her aunts and cousins.

Daughters of mothers with breast cancer have a higher than average breast cancer rate. In a comparison of sisters of probands whose mothers had breast cancer with sisters whose mothers had had no cancer, the respective frequencies were 14.6 and 2.8 per cent. The difference is barely significant statistically, but some of the noncancerous mothers had died early in life and might have developed cancer had they lived longer.

Mothers of breast cancer probands in our studies have had a higher rate of cancer of that site than have mothers of control probands. Sisters of controls need further checking before conclusions can be drawn concerning them, although the data now support the opinion that the sisters of cancer probands face a higher than average risk.

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A GENETIC CONCEPT FOR THE ORIGIN OF MELANOMAS*

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The genetic concept for the origin of melanomas discussed in this paper has been developed from the same genetic principles that underlie our present knowledge and understanding of the dynamics of natural populations and the origins of species (Demerec, 1955).

It has been shown repeatedly since 1931 that genetic influences are paramount in the etiology of spontaneous melanomas in platyfish-swordtail hybrids. Several geneticists, working quite independently, have agreed that any one of five multiple alleles of the dominant sex-linked macromelanophore gene of the platyfish, in combination with pigment-cell growth-modifying genes of related species, establish in a hybrid the physiological conditions that favor the development of melanoma.

Five dominant alleles of the potentially injurious macromelanophore gene are maintained in the eight natural populations of the platyfish (*Xiphophorus maculatus*) in southeastern Mexico and northeastern Central America (FIGURE 1). Of the 9000 adult wild platyfish collected from eight rivers, (Gordon and Gordon, 1957), 1879 had one or more macromelanophore patterns. However, not one wild platyfish of the 9000 examined had a visible pigment-cell abnormality. The macromelanophores are nevertheless potentially injurious cells since, in matings between *spotted* platyfish from different natural populations, a condition of melanosis develops from the atypical growth of pigment cells in the spotted intraspecific hybrids (FIGURES 2a and 2b). In matings involving a normal macromelanophore-patterned platyfish and a normal swordtail (*Xiphophorus helleri*), the atypical pigment cell growth is further advanced so that a melanoma develops in the spotted interspecific hybrid (Gordon, 1951a), as shown in FIGURE 2c.

It has been shown also that each of the eight natural river populations of the platyfish is genetically distinct, exhibiting differences in frequency of inherited macromelanophore and micromelanophore patterns. Data from genetic analyses of the hybrids between members of different river populations indicate that modifying genes that influence the expression of macromelanophore and micromelanophore patterns also differ in frequency in the various natural populations. Such differences suggest differences in the frequencies of other genes. One of the most dramatic genetic differences found among some of the populations of the same species is the fact that Mexican platyfish have a chromosomal sex-determining mechanism in which the male is XY and the female XX, whereas the British Honduran platyfish males are YY and the females WY (FIGURES 3 and 4). All these genetic

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differences may be taken as evidence of incipient speciation in the platyfish. Nevertheless, Gordon and Gordon (1954) showed that the members of the various isolated platyfish river populations are essentially similar with regard to size and body proportions, so much so that on morphologic criteria they cannot be categorized as belonging to different subspecies.

There is stronger evidence for active speciation in the platyfish in the divergent effects of the macromelanophore genes in the hybrid offspring of members of differing populations. When spotted individuals from different rivers are bred together, the expression of the macromelanophore genes may be greatly altered. In some interracial hybrids the gene may lose all its visual effect. In others, on the other hand, the expressivity of the gene may be increased to such an extent that melanosis results (Gordon, 1951b). These reactions provide evidence that the frequencies of the macromelanophore genes are influenced by the frequency of other genes. For example, in a population in which the gene complex is such that the presence of a certain macromelanophore pattern frequently results in melanosis, the frequency of that macromelanophore pattern would be expected to be zero. Differences in frequency of macromelanophore genes thus appear to reflect

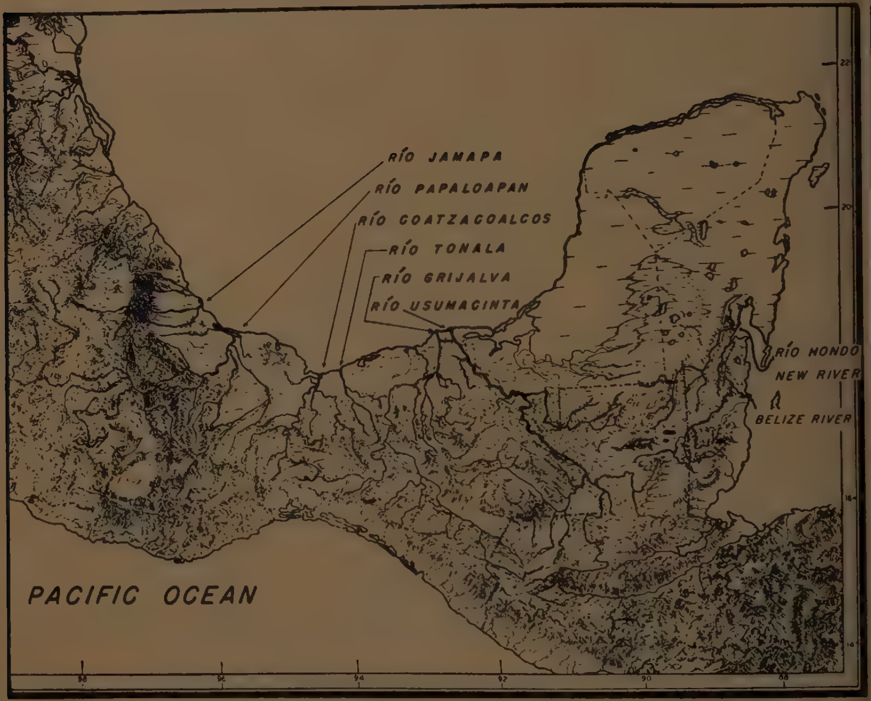

FIGURE 1. Part of Mexico, Guatemala, and British Honduras, showing the eight river systems at which *Xiphophorus maculatus* were taken. The New River is located just south of the Río Hondo. Reproduced by permission of the *Journal of Genetics* (Gordon and Gordon, 1957).

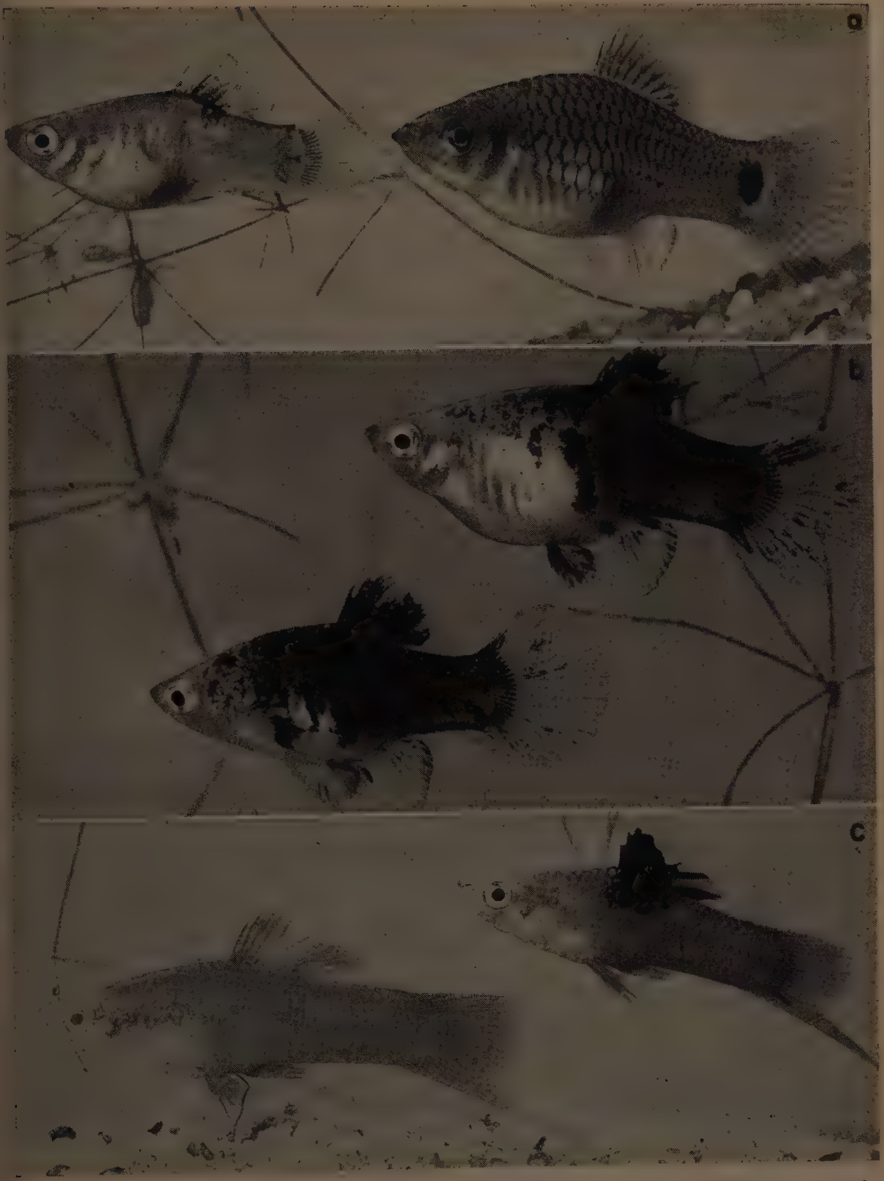

FIGURE 2. (a) Male and female platyfish (*Xiphophorus maculatus*). The male, to the left, originated in a stock obtained from the Río Coatzacoalcos. Note the spotted-dorsal pattern, *Sd*. The female, to the right, originated in a stock from the Río Jamapa. The female has the striped-sided pattern, *Sr* (see FIGURE 1). (b) Third-generation platyfish offspring of the original pair shown in 2a. Note the extreme extension of the spotted-dorsal pattern. Some degree of melanosis in these intraspecific hybrids is evident, for part of the dorsal fin has been destroyed. (c) Two platyfish-swordtail hybrids. On the left, an albino hybrid with the spotted-dorsal gene. Note that a small part of its dorsal fin has been destroyed, even though no black pigment cells are visible. On the right, a black-eyed platyfish-swordtail hybrid with a typical melanoma of the dorsal fin. Reproduced by permission of *Cancer Research* (Gordon, 1951a).

differences in the gene complexes of the various populations as well as environmental differences. There appears to be a balance of genetic and environmental forces that maintains the macromelanophore patterns in the population, thereby securing their favorable effects, perhaps with relation to predators, and that inhibits the occurrence of melanosis. In interracial hybrids the balance of genes modifying pigment-cell growth is upset. These crosses reveal a degree of genetic isolation as well as the possibility that both the production of melanosis and the suppression of macromelanophore pattern may be regarded as reducing viability. This leads to the thought that the macromelanophore mutation, when it first appeared in the wild

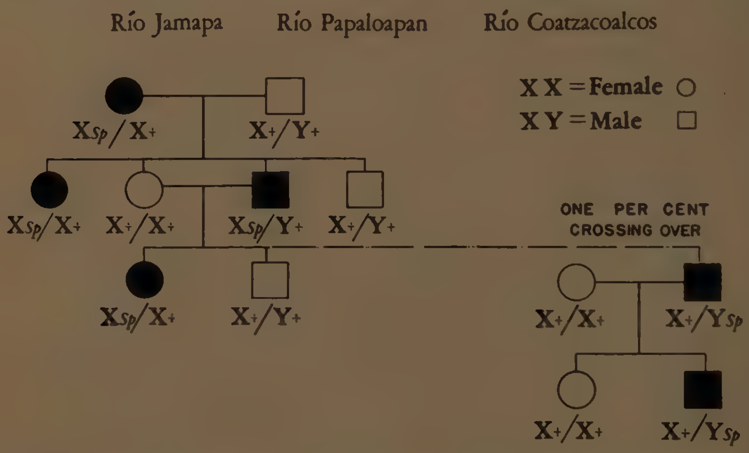

Figure 3. Diagram showing the unequal distribution of macromelanophore patterns between the sexes in the offspring of crosses between platyfish from populations with the XY-XX type of sex-determining mechanism. The Río Jamapa, Río Papaloapan, Río Coatzacoalcos, and Río Grijalva populations have this type of sex determination. Reproduced by permission of the *Journal of Genetics* (Gordon and Gordon, 1957).

platyfish, was probably harmful and upset the organism's established genetic balance; this, in turn, upset the biochemical processes involving pigment-cell growth.

It is probable that, prior to the macromelanophore mutation's successful establishment in the wild platyfish populations, other mutations that were to serve, in part, as modifiers and controllers of the growth and differentiation of macromelanophores must have accumulated first. Then, when the macromelanophore mutant reoccurred, these modifiers neutralized its lethal effects. In other words, the platyfish had to be made ready genetically before "accepting" the macromelanophore mutation, and this was accomplished by the platyfish by first accumulating macromelanophore-controlling modifying genes. When these prior genetic adjustments were incorporated in the genetic structure of the population, the platyfish accepted macromelanophore patterns, and this permitted it to develop into the polymorphic species it is. Much later, however, when a genetically balanced *spotted* platyfish from one river was mated in the laboratory aquarium with another normal member from another river, the new recombination of genetic modifiers of macromelanophore growth was not necessarily in balance in the *spotted* hybrids; in these individuals the large pigment cells grew atypically (FIGURES 2a and 2b).

The severity of atypical pigment-cell growth in hybrids between members of two geographically distinct platyfish populations was found to be not as great as in the interspecific hybrid fishes (FIGURE 2c). Gordon (1949, 1950) suggested that this may be so because the polygenic modifiers of macromelanophore growth in members of isolated populations of the same species are not as divergent in their effects as those that occur in the members of close but distinct species.

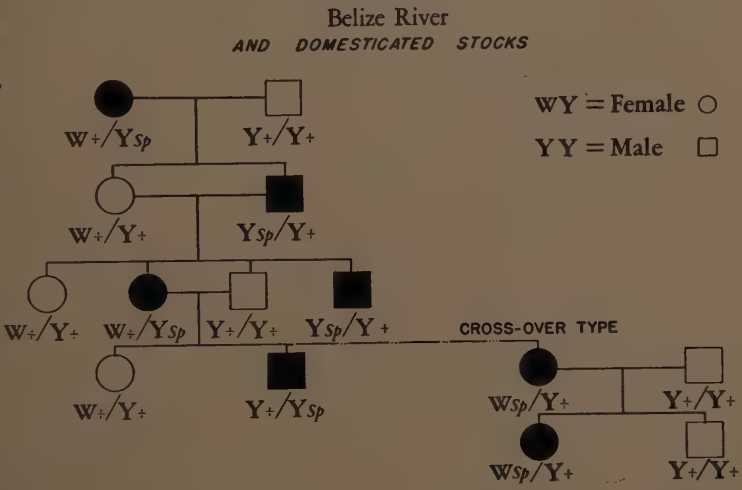

FIGURE 4. Diagram showing the unequal distribution of macromelanophore patterns between the sexes in the offspring of crosses between platyfish from populations with the WY-YY type of sex-determining mechanism. The Belize River population has this type of sex determination, as do the populations in the Río Hondo and the New River, just to the north of the Belize River (see FIGURE 1). Reproduced by permission of the *Journal of Genetics* (Gordon and Gordon, 1957).

Hybridization studies in mice representing different domesticated, inbred populations have shown a comparable reaction. Cloudman and Little (1936) indicated that sarcoma of the uterus was not found either in members of the *dba* or the short-tailed strains of *Mus musculus*, but when representative members of each strain were crossed, sarcoma of the uterus was found in a significant number of the first-generation hybrids. Later, Little (1939) showed that when a member of *Mus bactrianus*, a small, slow-maturing, relatively infertile species with no record of nonepithelial tumors, was mated with a member of the JAX C 57 *Mus musculus*, a large, rapidly maturing, fertile species susceptible to tumors, the incidence for both epithelial and nonepithelial tumors in their first-generation hybrids increased. Heston (1950), in a discussion of parallels in fish and mouse cancer genetics, revealed that in his experimental colony ten mice with tumors of the Harderian gland had been found, and that all ten were hybrids from a specific cross between members of the C3H and C57 black strains. While the Harderian gland tumor is rare, its occurrence in these specific hybrids and not in either parental strain or any of the other strains of the colony is noteworthy. Andervont (1941) showed that the status of complete resistance of parents to sarcoma 37, each parent representing a divergent strain, gives no assurances of the degree of resistance of their hybrids; in one experiment 87 per cent of the F_1 were susceptible. Tumor growths in plant hybrids also may be explained in terms of genic imbalances.

In xiphophorin fishes, the evidence is convincing that specific genes, through their interactions, are the primary initiators of the spontaneous development of melanomas. This evidence is strengthened by other genetic experiments that showed that the *site* of the body in which the melanoma will develop in the hybrid may be predicted. If a normal platyfish carrying the macromelanophore allele *Sd* (for a spotted dorsal fin) is mated with a swordtail, the platyfish-swordtail hybrid develops a melanoma of the dorsal fin. If a normal platyfish carrying the *Sb* allele (for spotted belly) is used in a similar mating, the platyfish-swordtail hybrid develops a melanoma along the mid-ventral surface of its body. If the *N* allele (or, under some conditions, the *Sr* allele) is inherited by the hybrid, it develops a melanoma of the flanks. If the *Sp* allele is present in the hybrid, the melanoma develops anywhere on the body.

Not only is the site of the development of a melanoma predictable in many instances on the basis of genetic principles, but so is the degree of the melanoma's pigmentation and its degree of malignancy. Breider (1938) and Gordon (1948) have shown independently that when the *albino* swordtail (one carrying the recessive gene for albinism, *a*) is used as one of the parents, and a macromelanophore-gene carrying platyfish is the other parent, the F_1 hybrids are melanotic. When these F_1 melanomatous hybrids (now heterozygous for albinism, *Aa*) are backcrossed to an albino swordtail (*aa*), some of their backcross (*aa*) hybrids develop *amelanotic* melanomas. Greenberg *et al.* (1956) studied the cytochemical and cytological differences between the melanotic and amelanotic melanocytes obtained from tissue cultures of both types of melanomas. They found that the albino gene, *a*, inhibited the synthesis of pigment beyond the stage in which the 5,6 dihydroxyindole compound is formed. In melanotic melanocytes carrying the dominant allele *A*, the oxidation of this compound continues to the quinone state, but in amelanotic melanocytes this step was inhibited. This phenomenon was correlated with cytological studies that revealed that the amelanotic melanocytes had more of the chief characteristics of neoplastic cell growth; they had fewer dendritic processes and less defined shapes. The amelanotic melanocytes were less able to differentiate into macromelanophores and thus to reach maturity. Amelanotic melanocytes grew more readily in tissue cultures and had a greater incidence of nuclear aberrations, such as hyperchromatism, giant and bizarrely shaped nuclei, and larger and more multiple nucleoli. Thus, genetic studies correlated with cytochemical analyses indicated that melanin cannot be considered an important factor in melanoma formation. Actually, the absence of melanin in melanomas is an indication of greater malignancy and indicative of a tumor cell population that consists mostly of melanocytes, which are now regarded as incompletely differentiated cells.

More recent correlative studies utilizing micrurgical and high-speed centrifugation techniques by Greenberg *et al.* (1958) indicated that the amelanotic melanoma cells had a relatively lower viscosity; their granular inclusions were finer and less numerous than in the melanotic melanocyte. Upon centrifugation, the amelanotic cells stratified more readily. Micrurgical manipulations by microneedles indicated that the cell membranes of amelanotic melanocytes were more friable, offered less resistance to the penetration of the needles, and had less dense cytoplasmic consistencies. These additional peculiarities of amelanotic melanocytes also may be attributed to the pleiotropic action of the albino gene interacting with an appropriate constellation of other genes, the principal one being the sex-linked gene for the presence of macromelanophores.

Humm and Young (1955) have demonstrated experimentally that the pro-pigment cells or *melanoblasts* originate in the neural tube of platyfish-swordtail hybrid embryos prior to stage 22. After the melanoblasts migrate to definitive areas of the body, they develop dendritic processes and the capacity to synthesize melanin; in their second stage of differentiation these same pigment-forming cells are called *melanocytes*. In terminal sites in dermal tissues the melanocytes, which are 10 to 100 μ in size, become sessile, round up, increase in size, and eventually differentiate into true melanophores (Marcus and Gordon, 1954; Ermin and Gordon, 1955). Melanophores, being at their terminal stage of differentiation, become pigment-effector cells measuring 100 to 470 μ (FIGURE 5). Eventually, melanophores become senile and degenerate; their cellular debris is phagocytosed by true macrophages and eliminated (Gordon and Lansing, 1943).

Observations from sites of transplantations (that is, from blastemas following amputation of melanomas) indicate that melanophores are normally replenished by proliferating and differentiating melanocytes. However, in the life history of genetically susceptible organisms, neoplastic melanocytes apparently do not differentiate into melanophores. As incompletely differentiated cells, the neoplastic melanocyte's life span and proliferative capacities are radically extended (Gordon, 1957).

From Chavin's work (1958) it would appear that the adrenocorticotropic hormone has the special property of melanogenesis. ACTH, then, may be responsible for the stimulus that normally enables melanocytes to differentiate into melanophores, thus producing the effect called melanogenesis. If this can be substantiated, then the genic imbalance in melanoprone individuals may be attributed in part to a genetically controlled inability of some individuals to produce an available supply of ACTH that would stimulate the melanocytes to transform into completely mature and differentiated pigment cells.

In this area of research the xiphophorin fishes have a distinct biological advantage as experimental animals because they have a specific, sex-linked

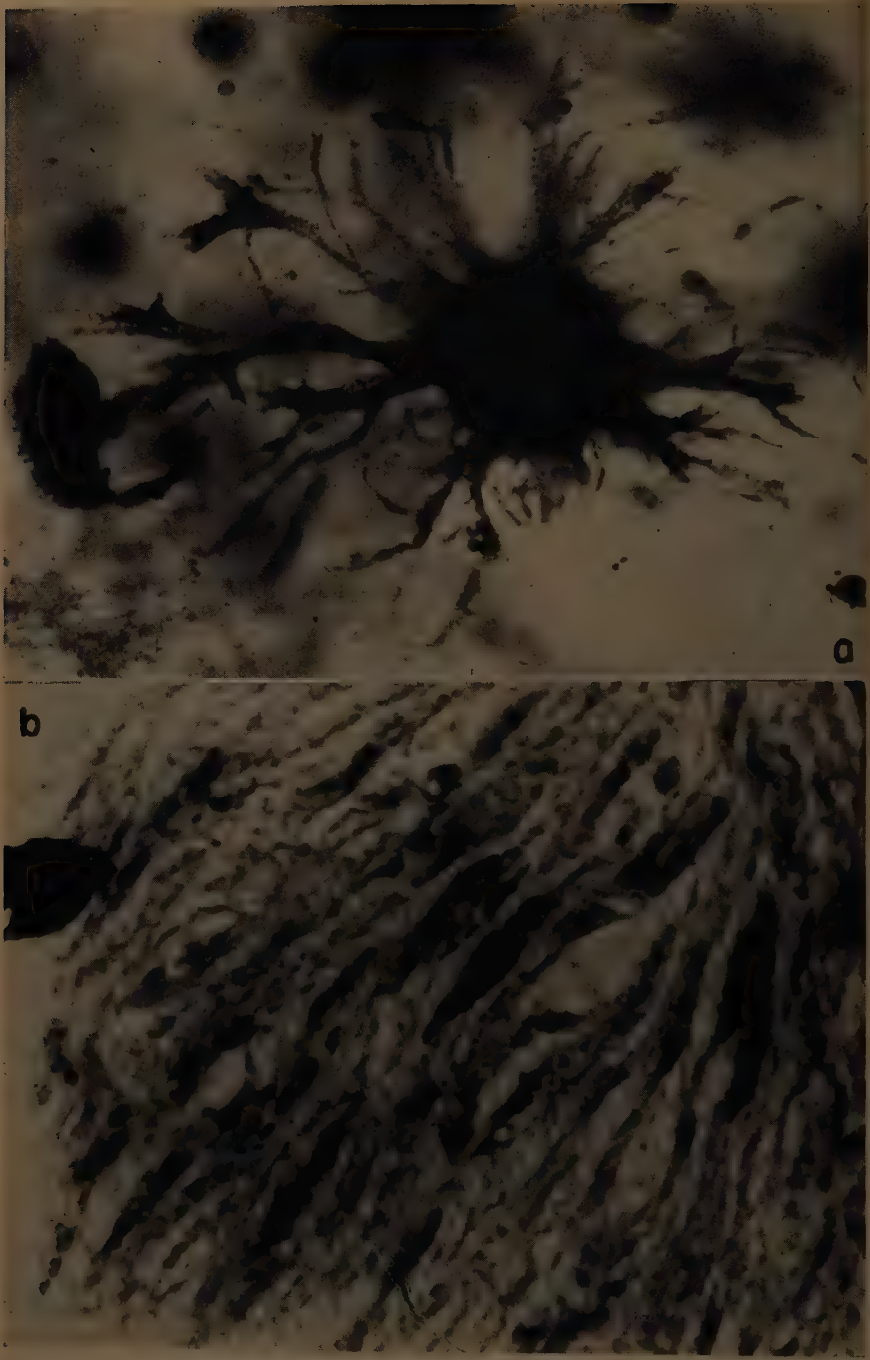

FIGURE 5. (a) Living macromelanophore in a tissue culture medium. Reproduced by permission of *Cancer Research* (Grand *et al.*, 1941). (b) Section through a melanotic melanoma of a platyfish-swordtail hybrid showing a "sarcomatous" arrangement of melanocytes. Feulgen stain; ×1200 (Gordon and Smith, 1938).

gene that determines the presence of a specific type of cell, a pigmented one whose growth pattern may be studied in a variety of genotypes. The growth of the macromelanophores may be modified genetically. The pigment cells may be induced to grow atypically and produce melanomas in response to the genetic imbalance created by a system of matings between members representing different natural populations or members representing different species. The critical physiological processes involved in the etiology of melanoma cells, which are under strict genetic control, are probably those that block the differentiation of melanocytes to their final maturation. At any rate, that is our present working hypothesis. It is now being tested experimentally.

Acknowledgment

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THE GENETIC BASIS OF ORGANIC FORM

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The problem of organic form is fundamental in biology. Form is a distinctive feature of all living things. It provides most of the characters that make possible the recognition and classification of organisms, and it enables us to trace the course of evolution. Darwin well remarks in *On the Origin of Species by Means of Natural Selection* that "morphology is the very soul of natural history."

The problem of form, however, is pre-eminent, not only because of its bearing on problems of taxonomy and phylogeny, but for a still more important reason. Form results from a precise correlation of growth processes and is the visible manifestation of that coordinating and integrating quality in organisms commonly known as biological organization. Morphology, not merely descriptive or comparative, but dynamic and developmental, must be the concern of all biologists. It is obviously of great significance for the problem of cancer, since cancer is a relaxation of the orderly control of development by which form is produced.

To account for the origin of organic form, and thus for biological organization, is obviously a problem of the greatest difficulty. Biochemical analysis of many metabolic activities has made great progress in recent years but, in comparison with these, our understanding of morphogenetic mechanisms is still very limited. Although Roux's *Archiv* now fills sixteen feet of library shelf, we know little more about the basic processes of morphogenesis than did Darwin or Mendel. In some quarters there is a disposition to avoid these problems as so hopelessly difficult that they cannot be approached until biochemical knowledge is much more complete. One can understand this attitude, but it is certainly important for all biologists to remember that the problem of organized and self-regulated development, most conspicuous in organic form, still stands at the very center of the life sciences.

That aspect of the form problem that involves its inheritance has been given relatively little attention. Most geneticists have been occupied with the more immediate questions of the physical basis of heredity, the nature of the gene, and the biochemistry of gene action. In many cases, to be sure, some genetic analysis of form has been made. Many of the characters studied in *Drosophila* are morphologic ones. Among plants, Hutchinson (1934) and Silow (1939) have worked on the inheritance of leaf shape in cotton, Frets (1955) on seed shape in beans, Lindstrom (1928) on fruit shape in tomato, and Anderson (1939) on corolla form in tobacco. Many more could be cited. In some cases linkage relationships between genes for shape and genes for other characteristics have been found (Lindstrom). In most of these studies, however, relatively little attention has been given to the question of how form is genetically determined and the mode of action of the genes involved. My purpose in this paper is to bring together some results from a study of this problem as it is exemplified in one family of plants, and to discuss their bearing on the problem of gene action.

Fruits of several species of the Cucurbitaceae, notably *Cucurbita pepo* and *Lagenaria siceraria*, provide favorable material for such a study. In both species there is a wide range of shape types, and it is possible to develop pure races for any particular type. The fruits are large and measurements can be made during almost the whole course of their development. At maturity most become firm enough to be preserved indefinitely for study. The plants are monoecious and usually cross pollinated in nature, so that it is necessary to inbreed them for several generations before beginning experimental work.

Evidence will here be presented for the conclusion that, at least in this material, genes are present that control form directly and not through their effects on individual dimensions. This evidence comes from several sources in experiments with *C. pepo* (Sinnott, 1935):

(1) Many fruit-shape differences show typical Mendelian inheritance. If a flattish type—disk or "patty-pan"—is crossed with one of the isodiametric or spherical lines, disk shape is completely dominant in F_1, and the F_2 shows three fourths disk and one fourth sphere, indicating a single gene difference between the two.

In a somewhat more complex case the same sphere as before was crossed with another approximately isodiametric race. The F_1 from this cross has a disk shape. When this is selfed, the F_2 segregates into nine sixteenths disk, six sixteenths isodiametric, and one sixteenth that has fruits considerably longer than wide. In this case the parental types evidently differ by two genes that are complementary in their effects, the double dominant producing a disk and the double recessive an elongate shape. This suggests that there are two flattening genes that are cumulative in their effect, both together producing extreme flattening, either one alone producing much less and, in the absence of both, no flattening at all. The correctness of this conclusion was proved by the results of various test crosses.

Other cases are more complex and evidently involve many genes. Some of these tend to flatten the fruit, others tend to inhibit flattening or to produce an elongate shape, and others tend to modify the pattern in various ways, as by the production of teeth, lobes, ridges, or necks (Sinnott, 1930).

(2) The shape of the fruit is inherited independently of its size. In a certain disk-sphere cross between which there is a single gene difference, the parental disk race was considerably larger than the sphere (1305 gm. as compared with 703 gm.). The F_1, having the dominant disk shape, was approximately the geometric mean between the two parental races in weight. In the F_2 the average weight of both disks and spheres was the same and approximately that of the F_1 (FIGURE 1). This suggests that fruit size is inherited in a typical polygenic fashion, but that there is segregating here a single gene that controls shape but is quite independent of size in its inheritance. The same result was found in more complex cases of shape inheritance.

(3) An analysis of dimensional inheritance supports this hypothesis. The F_2 segregation for shape index (ratio of length to width) is sharp and com-

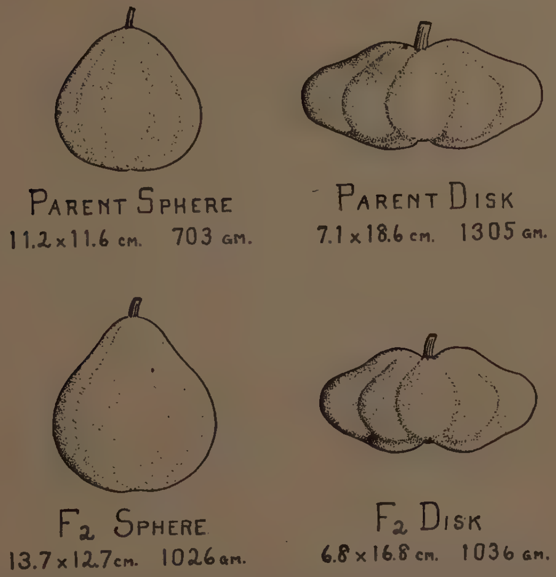

PARENT SPHERE
11.2×11.6 CM. 703 GM.
PARENT DISK
7.1×18.6 CM. 1305 GM.
F2 SPHERE
13.7×12.7 CM. 1026 GM.
F2 DISK
6.8×16.8 CM. 1036 GM.

FIGURE 1. Independence of shape and size. *Above*, parental types, with mean weights and dimensions of sphere and disk parents. The F_1 was disk and about the geometric mean in weight between the two parent types. In F_2, *below*, the spheres and disks segregate sharply as to shape, but the mean sizes are essentially equal and like that of the F_1.

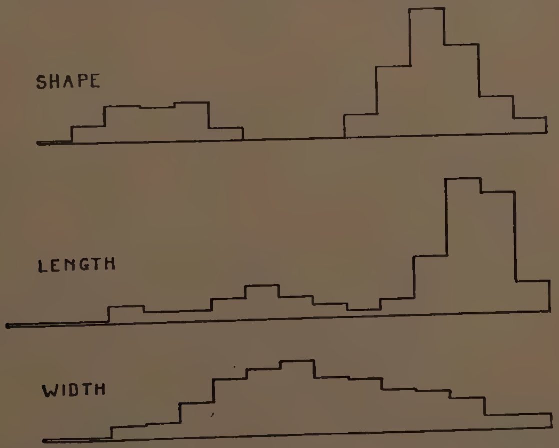


FIGURE 2. Segregation of shape index, length, and width in F_2 of the cross between disk and sphere shown in FIGURE 1.

plete. That for length shows a bimodal curve, and that for width an essentially smooth one (FIGURE 2). This might be interpreted as the result of a single gene determining length, and of many determining width. The far sharper segregation for shape index, however, indicates that this is the trait that is under direct genetic control, and that a given dimension results from a particular shape superposed on a particular inherited size.

(4) In more complex F_2 segregations the relationships between the dimensions are what they would be if genes controlled shape indices and not the dimensions themselves. In crosses where the parental types differ in both shape and size of their fruits there are some size differences in the parental races and the F_1 because of environmental factors. Since shape is constant in these types, length shows a close *positive* correlation with width. In each of twelve segregating F_2 populations, however, length and width are *negatively* correlated (TABLE 1). This is to be expected, for if there is a certain amount of material available for the growth of a given fruit, as the result of its genotype for size, and if genes that govern the distribution of this material are independent of size, then the longer a fruit is, the narrower it will be, and *vice versa*.

TABLE 1

COEFFICIENTS OF CORRELATION BETWEEN LENGTH AND WIDTH OF FRUIT IN SIX REPRESENTATIVE LINES AND IN THE F_1 AND F_2 PROGENIES OF FIFTEEN CROSSES BETWEEN LINES DIFFERING IN FRUIT SHAPE

Line	No. of individuals	Correlation coefficient	Line	No. of individuals	Correlation coefficient
103	156	+.57 ± .04	438	83	+.55 ± .05
125	84	+.73 ± .05	C	136	+.56 ± .06
391	193	+.54 ± .05	M	105	+.91 ± .01
F_1			F_2		
1-21	72	+.48 ± .10	1-22	220	−.44 ± .06
2-21	39	+.52 ± .12	2-22	231	−.37 ± .06
1-22	32	+.41 ± .15	1-23	392	−.32 ± .05
3-22	118	+.62 ± .05	3-23	430	−.41 ± .04
4-22	93	+.61 ± .07	4-23	200	−.71 ± .04
6-22	23	+.25 ± .11	6-23	236	−.12 ± .06
7-22	55	+.39 ± .12	7-23	239	−.45 ± .05
8-22	90	+.26 ± .10	8-23	535	−.33 ± .04
9-22	42	+.13 ± .15	9-23	303	−.19 ± .05
3-23	14	+.58 ± .18	3-24	290	−.40 ± .05
17-23	30	+.53 ± .13	17-24	275	−.47 ± .05
19-23	14	+.30 ± .24	19-24	364	−.43 ± .05
20-23	32	+.32 ± .16	20-24	273	−.36 ± .05
23-23	32	+.59 ± .12	23-24	135	−.22 ± .07
25-23	16	+.79 ± .12	25-24	302	−.30 ± .05

(5) The variability of length and of width is about the same in the pure types and the F_1 generations, but length in the F_2 is about *twice* as variable as width (TABLE 2). This follows from our hypothesis, since fruits are radially symmetrical, and width thus enters twice into the determination of volume (w^2l), while length enters only once. A given change in width will therefore produce a change twice as great in length if genes control shape independently of size.

(6) These shape differences are far more complex than can be represented by the ratio of length to width or between any two dimensions. Each type

TABLE 2

COEFFICIENTS OF VARIATION FOR FRUIT LENGTH AND FRUIT WIDTH IN SIX REPRESENTATIVE LINES AND IN THE F_1 AND F_2 PROGENIES OF FIFTEEN CROSSES BETWEEN LINES DIFFERING IN FRUIT SHAPE

Line	No. of ind.	C.V. of length	C.V. of width	Line	No. of ind.	C.V. of length	C.V. of width
103	156	12.4 ± 0.7	10.0 ± 0.6	438	83	20.3 ± 1.6	20.0 ± 1.6
125	84	8.6 ± 0.7	8.8 ± 0.7	C	136	14.6 ± 0.9	12.9 ± 0.8
391	193	13.2 ± 0.7	18.2 ± 0.9	M	105	16.6 ± 1.2	17.8 ± 1.2
F_1				F_2			
1-21	72	13.0 ± 1.1	14.2 ± 1.2	1-22	220	35.7 ± 1.7	16.6 ± 0.8
2-21	39	12.0 ± 1.4	12.4 ± 1.4	2-22	231	27.5 ± 1.3	14.7 ± 0.7
1-22	32	11.1 ± 1.4	8.8 ± 1.1	1-23	392	34.2 ± 1.2	17.9 ± 0.7
3-22	118	13.5 ± 0.8	11.4 ± 0.8	3-23	430	36.0 ± 1.2	19.7 ± 0.7
4-22	93	12.3 ± 0.9	10.6 ± 0.8	4-23	200	44.4 ± 2.2	24.6 ± 1.2
6-22	23	8.0 ± 1.2	14.5 ± 2.2	6-23	236	34.0 ± 1.6	15.8 ± 0.7
7-22	55	8.5 ± 0.8	10.6 ± 1.0	7-23	239	36.8 ± 1.7	15.5 ± 0.7
8-22	90	8.2 ± 0.6	6.6 ± 0.5	8-23	535	31.9 ± 1.0	14.9 ± 0.5
9-22	42	7.5 ± 0.8	7.2 ± 0.8	9-23	303	34.8 ± 1.4	15.8 ± 0.6
3-23	14	11.4 ± 2.1	6.0 ± 1.1	3-24	290	34.9 ± 1.5	16.6 ± 0.7
17-23	30	6.6 ± 0.9	7.3 ± 0.9	17-24	275	31.2 ± 1.3	18.5 ± 0.8
19-23	14	7.8 ± 1.5	8.2 ± 1.6	19-24	364	35.1 ± 1.3	18.6 ± 0.7
20-23	32	8.4 ± 1.1	9.5 ± 1.2	20-24	273	34.3 ± 1.5	16.8 ± 0.7
23-23	32	9.3 ± 1.2	7.7 ± 1.0	23-24	135	29.0 ± 1.8	14.0 ± 0.9
25-23	16	11.3 ± 2.0	9.9 ± 1.8	25-24	302	37.5 ± 1.5	19.4 ± 0.8

has a special pattern of fruit shape of its own. This may involve an elongate neck, as in the spoon gourd; a transverse constriction, as in the bottle gourd; or equatorial teeth of various sizes, as in the patty-pan or the crown. These specific patterns tend to reappear in segregating populations as though inherited as wholes. The pattern seems to be an organized entity, each part of which is related to the rest, as in the organic forms inscribed in rectangular coordinates by D'Arcy Thompson.

These various lines of evidence point to the conclusion that in this material there are genes that directly determine form and do not operate by controlling individual and independently inherited dimensions. If the latter were the case the facts here presented would be much more difficult to explain. The specific patterns would tend to disintegrate. There would be far more combinations of maximum length with maximum width and thus a greater range of fruit size than is actually found. A number of workers, however, have postulated independent dimensional control, notably Anderson (1939) in *Nicotiana* corollas and both Frets (1955) and Malinowski (1921) in bean seed shape. It has also been suggested that linkage between dimensional genes may determine shape. The data presented to support these conclusions could be explained more simply, in my opinion, on the assumption that there are genes controlling shape directly.

In the cases of fruit shape inheritance just discussed, it is the character of the mature fruit that has been analyzed. The shape genotype evidently must control the entire process of fruit development, however. A study of this process provides important information about shape determination.

In most races of *C. pepo* the shape of the mature fruit differs but little from that of the early ovary primordium. In other genera, and especially the more extreme gourd shapes in *Lagenaria* and *Trichosanthes*, however, shape changes markedly as the fruit develops. In some, length grows faster than width, and the fruit becomes relatively more elongate; in others, the reverse is true. In every such case examined, the *relative* rate is constant, so that if length and width of the enlarging fruit are plotted on a double logarithmic grid, the points fall along a straight line and the allometric constants for level and slope can be determined (Sinnott, 1936). In FIGURE 3 are shown the allometric lines for length and width in a number of types.

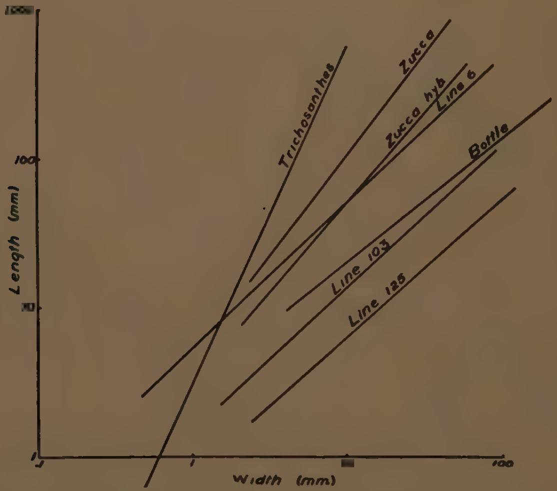


FIGURE 3. Length and width, plotted logarithmically, in growing fruits of several cucurbit races, showing differences in relative growth of the dimensions.

Within the same species, races may differ markedly in the slope of this line and thus in the value of the constant *k*. The zucca race grows considerably faster in length than in width (approximately 1.3), but the bottle variety grows more slowly (approximately 0.8). If the two are crossed, the slope of the line in the F_1 is much like that in the zucca parent, but in the F_2 there are two groups of plants: those with lines like the zucca (more numerous) and a smaller number like the bottle (FIGURE 4). This suggests a single-gene segregation, and indicates that the allometric constant *k* is the character that is segregating.

It is evident that in cases such as this, where the relative growth constant is greater or less than 1, the shape of the fruit at maturity will be affected by the size to which it grows. Fruits of plants with the same genotype for shape (relative growth) but a different one for size will not have the same fruit shape at maturity. In the F_2 of the cross just described there was no sharp shape segregation at maturity, since this was masked by segregation for size. Only when the relative growth constants for each plant were determined could a genetic analysis for shape be made.

It should be remembered, of course, that it is not only the relation of length to maximum width that shows allometric constancy, but all the other ratios of the pattern as well. In the bottle gourds, for example, the ratio between fruit length, on the one hand, and the width of the upper lobe, the width of the constriction, and the width of the lower lobe, on the other, all show allometric growth, but with slightly different constants. In other words, the entire pattern of the developing fruit is constantly changing.

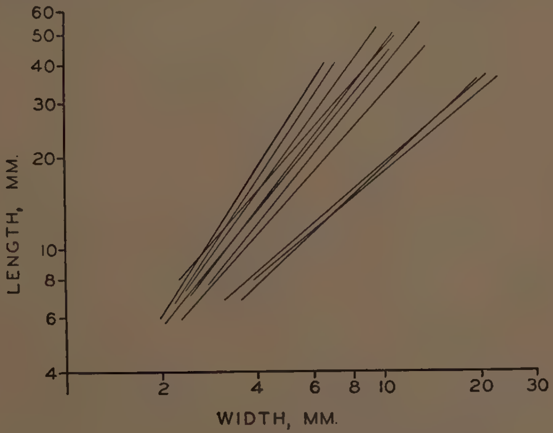
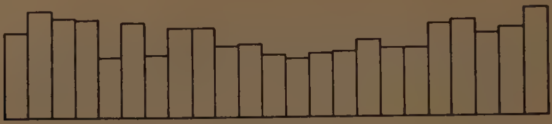


FIGURE 4. Relative-growth lines for length and width in F_2 of a cross between the zucca and bottle in FIGURE 3, showing segregation in slope of line, constant *k*.

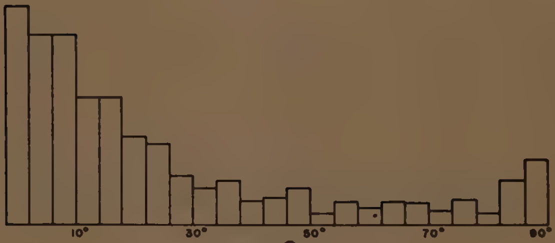
What histological changes, one may ask, are associated with these different dimensional growth rates? Are they concerned with differences in the plane of cell division, direction of cell expansion, or other factors?

As in most plant structures, the form of the organ here is not related to the form of its cells. In both the elongate and the flat types the cells of the fundamental tissue are essentially isodiametric. The planes in which the cells divide, however, are different in the two. In young ovaries of a number of shape types, whenever cells were found in mitosis, measurements were made of the angle between the axis of the mitotic figure and the axis of the ovary (FIGURE 5). In elongate types there was a preponderance of low angles, indicating divisions at right angles to the ovary axis, and thus greater growth in length. In types where length and width grow equally, angles were divided nearly equally between 0° and 90°, showing that the cells were dividing in all planes. Where the angles were grouped according to the stage of mitosis, grouping was sharper at telophase than at anaphase, and sharper at anaphase than at metaphase, indicating that the mitotic figure changes its orientation somewhat before settling down to its final position just prior to wall formation (FIGURE 6). In vacuolate plant cells, the position of the future division wall is occupied by a cytoplasmic diaphragm, suggesting that the plane of division, and thus ultimately the direction of growth, is determined early in the cytoplasmic body and not by the orientation of the mitotic figures. This is further shown by the fact that during prophase the mother cell elongates slightly at right angles to the plane of future division (as can be shown where almost all the divisions are in one plane, as in *Trichosanthes*), as though the relative directions of growth were determined in the organized structure as a whole rather than in the individual cells.



LINE 391

LENGTH AND WIDTH GROW EQUALLY



LINE CL

LENGTH GROWS FASTER THAN WIDTH

FIGURE 5. Distribution of angles between the axis of mitotic figures and the longitudinal axis of ovary primordium (*above*) in a race where length and width grow almost equally and (*below*) in one where length grows considerably faster than width.

The evidence here presented warrants several general conclusions:

(1) In the material studied, which is presumably typical of plant forms generally, genes are acting to control fruit shape.

(2) These genes govern an entire specific pattern, an organic gestalt, and not the individual dimensions by which it may be described.

(3) This pattern becomes manifest, not simply in the mature fruit, but in its developmental history. What is controlled, and what segregates in inheritance, is a series of *relative* growth rates.

(4) The control of the specific directions that growth takes inheres in the entire living system and seems not to be the summation of processes determined by events in individual cells.

If these conclusions are correct, there remains the problem of determining the actual mode of action of these "shape" genes. We know little enough about gene action in general, and the processes these particular genes control seem more difficult to get at than those of biochemical synthesis that have claimed most of the attention of the students of the genetics of development. The action of shape genes is well worth attention, however, for these are concerned with the basic problem of organization itself. Something must coordinate the relationships between genes during development—not only genes for shape, but all others. If there are 30,000 in a fertilized egg, and each mediates the production of a specific substance, obviously something more than independent gene action is needed if a normal organism is to be formed. Developmental processes are so correlated that an orderly series of embryonic steps, not chaos, results.

Nothing more can be offered here than a few tentative suggestions as to how these shape genes act.

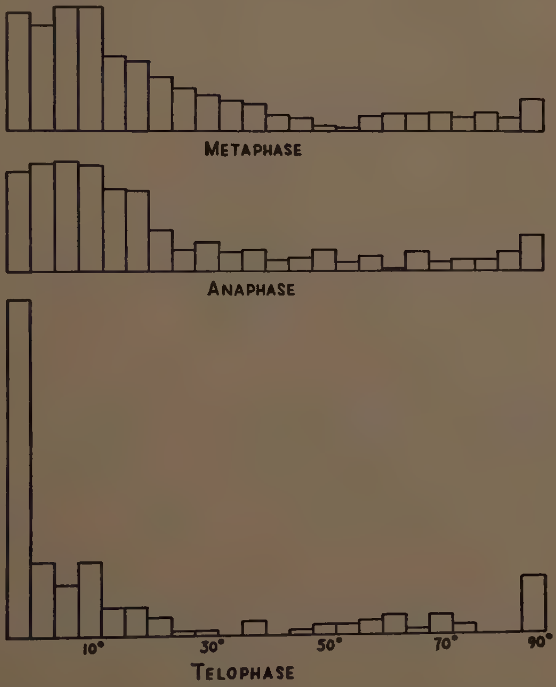


FIGURE 6. Distribution of angles between the axis of mitotic figures and the longitudinal axis of ovary primordium, in metaphase, anaphase, and telophase in *Trichosanthes*, a form where length grows much faster than width ($k = 2.2$). The figure does not assume its final orientation until late in mitosis.

(1) Shape genes may produce specific substances that modify the organized system of morphogenetic potencies in particular ways. If we imagine such a system to be analogous to a series of three-dimensional coordinates within which the shape of the fruit, in its various developmental steps, is inscribed, a chemical change might be expected to modify the system as a whole by changing the relationships of the coordinates (FIGURE 7) in such a way as D'Arcy Thompson has illustrated so vividly. It would be most unlikely that a gene-produced substance would change one part of a coordinated system without affecting, to some degree, all the rest by a simple sort of pleiotropy. This organized morphogenetic system must still be regarded,

on such a conception, as something given and still to be explained, a part of the larger problem of biological organization.

(2) More specifically, this morphogenetic system may be present in the cytoplasm and have its basis there in macromolecular patterns, as Schmitt (1956) and others have suggested; in specific topological relationships, as Rashevsky (1954) postulates; or in a series of diffusion reactions, such as Turing (1952) believes are operative. Any of these could be modified by gene-produced substances.

(3) It has been suggested by Goldschmidt (1938) and others that genes primarily control rates of physiological processes. For shape genes we may carry this idea considerably farther and assume that they control, not the absolute rates of these processes, but their *relative* rates—relative not only as to time but as to *space*. On such a conception a shape gene might be regarded, so to speak, as a unitary element of the organized developmental process. Those rate relationships compatible with one another would together constitute the organized pattern. Incompatibilities would be lethal. Perhaps the concept of genetic homeostasis proposed by Dobzhansky and Wallace (1953) and by Lerner (1954) might be extended to include this sort of regulation.

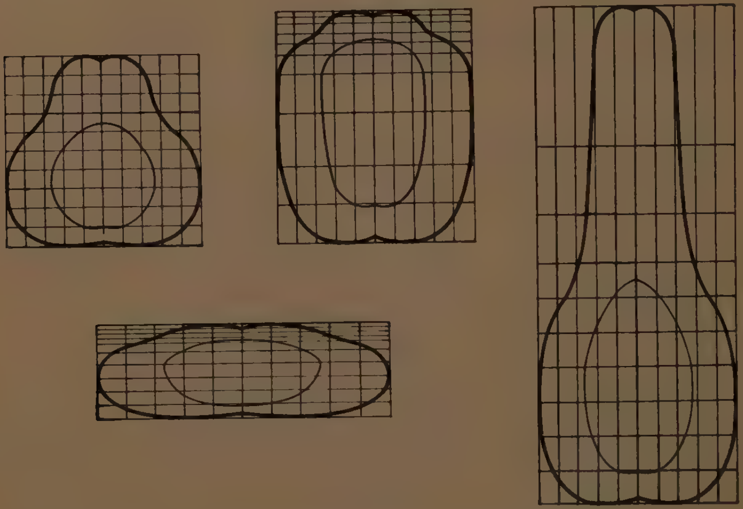


FIGURE 7. Forms of various fruit-shape types inscribed in rectangular coordinates, derived by transformation of the isodiametric type at the upper left.

Whatever the basis of the action of genes for shape, it must involve control of *relationships* and not simply of individual rates or substances. The mechanisms by which these developmental relationships are determined, and the organic patterns thus produced, are by no means clear. Some biologists have been intrigued by the possibility of deriving the characters of the organism directly from those of the specific protein molecules that distinguish it.

There are evidently serious difficulties in understanding the way in which relationships among the constituents of a molecule can be translated into those among the constituents of an organism, although it has sometimes been suggested that the organism should actually be regarded as a sort of macromolecule.

The problem of biological organization is obviously involved in all this. If the essential quality of life is reproduction, a statement often made today, this seems not to be simply the continued duplication of specific nucleoproteins, the genes, but that wider sort of reproduction of an organized system through the action of these genes, such as is evident in embryonic development and differentiation, in regeneration, and in the various processes of self-regulation. The simplest manifestation of such a system, and the one that may turn out to be most readily accessible to investigation, is organic form.

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GENETICS OF HOMEOSTASIS AND OF SENILITY

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The fertilized egg in which man begins his existence, if it remains alive, is gradually but inexorably transformed into an embryo, infant, child, youth, adult, grayhead, and finally a corpse. This path or trajectory of the development is determined by the interaction of the genotype with the sequence of the environments in which the development takes place. It must be stressed that every portion of the trajectory is so determined—the upswing prior to maturity, the mature phases, as well as the decline, senility, and the inevitable dissolution. This simple point needs emphasis because we geneticists often speak and write as though the genes each determined a discrete "character" or "trait," particularly of the adult state. However, the development does not proceed by gradual accretion of traits shaped by individual genes independently from what has gone on before or from what other genes are doing. The development is a highly integrated process, which may be likened to a symphony in which the genes are members of an orchestra rather than soloists. The organism is sometimes spoken of as a time-binding machine. This is correct as far as it goes, but I prefer to say that the organism carries in it the history of its life. Its state at any given moment is determined by its preceding states, as modified by the changes in the environment and by the process of aging.

The environment does not, however, remain absolutely constant from one instant to the next, nor are the environments that different individuals face at a given instant ever absolutely identical. The impact on the organism of environmental changes, or of environmental differences, causes variations in the developmental trajectories. The fact of basic importance is that these variations are not always haphazard. Many of them, far too many to be due simply to chance, are adaptive. Some of them allow the organism to continue on a developmental trajectory that is very similar to that which would be followed if the impact of the environment did not occur. Others cause the development to be switched to an alternative path that fosters survival and reproduction. Walter Cannon named this ability of the organism to react adaptively to environmental changes "homeostasis," and described it very felicitously as the "wisdom of the body."

Homeostasis is frequently misunderstood to mean absence of change. The matter is much subtler. Homeostatic mechanisms maintain an approximate constancy only of those processes that are essential for the continuation of life and of development towards successful reproduction. However, the constancy of these processes is attained through modification of other processes. The maintenance of a constant body temperature is a good example. Our temperature is approximately constant despite variation of the temperature of the environment; this is possible because of such factors as changes in the state of the sweat glands and dilation or constriction of the superficial blood vessels. A genotype that would cause the sweat glands to function at a constant rate at different temperatures would not yield a homeostatic mechanism.

Furthermore, the "wisdom of the body" is not an inherent property of life that would permit the body to be wise in all environments and at all stages of the development. The wisdom is limited to those environments that the species has frequently encountered in its evolutionary history. No one has put this better than Schmalhausen. The body reacts to environmental changes that are "normal" in the above sense by adaptive modifications; it reacts to environmental agents that the species has rarely or never encountered in its history or to abnormal intensities or the customary agents by morphoses. Morphoses are adaptively ambiguous. They may be useful, or neutral, or definitely harmful. Substances to which our body reacts by very harmful morphoses are called poisons. We possess defense mechanisms against the ultraviolet, but not against higher energy radiations. Our homeostatic mechanisms function successfully within a certain range of temperatures, but we freeze at lower temperatures and suffer burns at higher ones. Modifications, as well as morphoses, are evidently conditioned by the genotypes of the reacting organisms; adaptive as well as inadaptive responses are equally within the norms of reactions of the human genotypes.

Homeostasis is not the gift of a good fairy, it is a product of natural selection. Homeostatic mechanisms have arisen and have become perfected in evolution because they increased the adaptive values of the genotypes that conditioned them. Natural selection is, however, opportunistic. It cannot adapt the species to environments that do not yet exist. The opinion of some philosophers that the actions of living things are always directed toward the future is not valid. When the environment of a species changes rapidly, as that of mankind has been doing for the last ten to twenty thousand years, the adaptedness of its genotypes lags behind the times. The biological function of human culture lies in the fact that the faculty for transmitting learned knowledge is a nimbler adaptive mechanism than the genetic change in the biological evolution is or can be. Nevertheless, the frequent bodily, and especially mental, breakdowns in civilized societies may mean that in our species the evolution of homeostatic mechanisms has lagged behind the biological need for them.

Another limitation of the homeostatic mechanisms is that some of them function most efficiently during youth and maturity and tend to deteriorate during the autumn of life. Senility brings frail health and a lowered capacity for recuperation after physical or mental exertion. This is as it should be if homeostasis is fashioned by natural selection. Indeed, the adaptive value of a genotype is measured by the contribution that its carriers make to the gene pool of the succeeding generation, in relation to the contributions of other genotypes in the same Mendelian population. It follows that the more precious an individual is to the species the greater the contribution to the reproductive function he is expected to make (this ignores, of course, the possibility that, in human societies, an individual may enhance the adaptive value of a population in ways other than becoming a paterfamilias). Natural selection, accordingly, is erecting most efficient adaptive mechanisms to operate during the springtide of life. Conversely, natural selection does not counteract the spread of genotypes that hasten the decay of the organism after the close of the reproductive age. Hence senility, with its "thousand natural shocks that flesh is heir to."

An objection may be raised that the above argument attempts to prove too much. After all, animals do not die immediately after their youngest progeny has been born, or when this offspring has reached maturity and become independent. Some animals manage to go on living for some time afterward. I like to use the following analogy to counter this objection. Some cheap watches are sold with a "guarantee" that they will function for a year, or for three months, or for some other period. However, most of these watches continue to function for some time—occasionally for a considerable period—after the expiration of the guarantee. Surely, this is not surprising, since a watch designed to stop running immediately after the expiration of the guarantee period would have to be a very precise mechanism, difficult and expensive to make. Some of us old-timers are, then, like the cheap watches that continue ticking beyond their guaranteed time. Natural selection could act to dispose quickly of the postreproductive veterans only if their continuing existence were injurious to the reproductive success of the younger members of the population. Even then, it would have to be a so-called second-order selection, which is likely to prove a rather inefficient and slow process. There is no stimulus for such a selection as long as the presence of the superannuated members of the population can in any way benefit the population as a whole.

The customary dichotomy of hereditary and environmental disease does not withstand a critical examination. Health and disease are conditions of the phenotype, not of the genotype. Any disease or infirmity is in a very real sense hereditary, since it represents a response of a human genotype to some environment. The common cold is a human disease, and it does not, as far as is known, afflict cats, or mice, or canary birds. It is perhaps less evident that any disease is also environmental. Hereditary diseases are not necessarily incurable; in principle, any deviation from an adaptive developmental path could be corrected by some treatment, although for many hereditary diseases such treatments are as yet unknown. Any "treatment" comes, of course, from the environment.

A distinction that is more meaningful than that between "hereditary" and "environmental" traits is the difference that exists between the adaptive norm of a species and its aberrant members. However, it must be stated at the outset that this distinction is not an absolute one and that intermediate situations occur. The adaptive norm of a species is composed of an array of genotypes. These genotypes share the common property of engendering developmental paths that are homeostatically buffered against environmental variations that occur more or less frequently in the habitats where the species lives. In populations of any species that is not on the verge of extinction, the adaptive norm comprises a majority of individuals. All "normal" and healthy persons are included in the adaptive norm of the human species. It is to be noted, however, that they are carriers of many different genotypes, not of only one "normal," "typical," or "ideal" one. Apart from the adaptive norm, the populations of probably all sexually reproducing species contain also some aberrant genotypes, which constitute the "genetic loads" of these populations. The genetic load consists of mutant genes and other genetic variants that incapacitate their carriers in various ways, and they do so in the environments in which the species usually lives. These environments are thus "normal" for it.

It should not be imagined that the carriers of the aberrant genotypes are always ailing, whereas the adaptive norm is always sound and strong. The former may benefit by living in certain special environments. On the other hand, no genotype possesses a preternatural ability to resist all environmental hazards. The carriers of genotypes that are adaptively superior under most conditions may be infirm in environments that are unhealthful for them. Most, perhaps all, people are susceptible to infection by syphilis, malaria, and some other pathogens and parasites. Pathogens and parasites are environmental agents, to which our species has not acquired in the process of its evolution a built-in resistance.

We may use an even cruder example. Man does not possess natural defenses against many kinds of mechanical injuries such, for example, as those that a turtle withstands easily when it withdraws into its carapace. I am not one of those who value consistency above common sense, but I submit that an analysis of the relationships between the genotype and the phenotype leads to some conclusions that, at first sight, may seem strange. Our genes make us less resistant to skin cuts and abrasions than are turtles or pachydermatous mammals. Some aberrant human genotypes are, however, even more sensitive. As shown by Dean, possessors of the human genotypes that include the gene for the disease known as porphyria suffer more frequent and more painful skin abrasions than do possessors of genotypes free of this gene.

The adaptive norm may thus yield infirm phenotypes in unfavorable environments with which the homeostatic mechanisms lack the capacity to cope. Another source of infirmity that is compatible with the possession of a superior genotype is the process of aging. As pointed out above, the phenomenon of senility is not inconsistent with high fitness in the evolutionary sense. Infirmities of old age are not necessarily discriminated against by natural selection. This may be regarded as the basic postulate of gerontological genetics. Senility and death are epilogues of the developmental paths engendered by the genotypes that constitute the adaptive norm of the species.

I must now digress briefly into *Drosophila* genetics. One of the salient characteristics of many *Drosophila* mutants is a high variability of their phenotypic expression, compared to that in the wild flies of the same species. For example, the eyes of normal flies vary relatively little in either shape or size, especially among individuals of about the same general body size. By contrast, the mutant eyeless has eyes varying all the way from none at all to almost normal in size, and, in addition, frequently quite different eyes on the two sides of the head. As a matter of fact, the mutants used in classic genetic experiments were hand-picked for relatively low variability, and especially for lack of phenotypic overlaps with the wild-type condition. Most mutants that arise in *Drosophila* cultures do not fulfill these desiderata, and usually they are dispatched down the drain.

More recent studies on natural populations of *Drosophila* have disclosed that homozygosis for many, in fact for most, chromosomes found in wild populations leads to losses of viability or of fertility (such homozygotes, carrying certain "wild" chromosomes in duplicate, can be obtained in laboratory experiments with the aid of certain techniques that cannot be considered here). A possible way to describe this situation is to say that most wild chromosomes carry various recessive mutants with deleterious effects in homozygotes. Now, one of the remarkable properties of these subvital homozygotes lies in the fact that their rates of survival are often quite variable and sensitive to changes in the environment. The adaptive norm of the species is, then, homeostatically buffered against a certain range of environmental variations. It has been made so by natural selection. Mutants and genotypes that carry in duplicate "wild" chromosomes are often deficient in homeostatic adjustment. These mutants and homozygotes are rare in nature; they do not belong to the adaptive norm of the species; natural selection has not equipped them with homeostatic defenses.

A basically similar situation is found when we consider the postreproductive stages of the adaptive norm in man. The condition known as "senility" is a highly variable one. Vigor and vitality may be retained until an advanced age, and may ebb only slowly. On the other hand, some bodily function may go conspicuously into decline. It may be the circulatory system, or the kidney function, or the brain, or a neoplastic growth may arise. Such a high and irregular variability is characteristic of developmental trajectories not directly controlled or regulated by natural selection. This does not mean, of course, that human variability during postreproductive years is independent of the differences between the various genotypes that constitute the adaptive norm of the human species.

As pointed out above, the concept of the adaptive norm does not by any means imply that all so-called normal men are either genotypically or phenotypically alike or react similarly to environmental variations. The adaptive norm is an assemblage of diverse genotypes. This diversity furnishes the creative leaven of human evolution. Far from being a curse, the diversity enhances the adaptive value of human populations. However, the genotypes that constitute the adaptive norm share an important property. They engender developmental paths that, during the prereproductive and reproductive phases, are homeostatically buffered against a certain range of environmental variations. Individuals may differ in body build, in physiological, biochemical, and psychological traits. Inasmuch as they belong to the adaptive norm, however, they are fit to survive in many kinds of environments. People can become accustomed to different diets, trained to do different work, acclimatized in different geographical regions, or can adopt different ways of life. The adaptive norm of our species is highly versatile.

The adaptability and plasticity are, however, reduced with age. The homeostatic buffering against environmental shocks is weakened during the postreproductive phase. The genetic situation, however, is extremely complex. Consider, for example, a genotype that is advantageous to its carriers during the prereproductive and reproductive periods. Such a genotype will be favored by natural selection even if it causes loss of health and suffering during the postreproductive life. Conversely, a genotype that lowers the reproductive fitness will be discriminated against by selection even if it is conducive to vigorous old age. The mean longevity in most human societies has risen sharply in recent times. As recently as 1838 the average longevity of London "gentlemen" was close to 44 years, of tradesmen 26, and of mechanics, servants, and laborers 24 years. Since only a minority of the population survived even to the end of the reproductive span, the pressure of natural selection had to be concentrated to secure the fitness of the prereproductive and the early reproductive periods.

Human social life introduces further complications. Haldane has pointed out that natural selection may either favor or hinder the prolongation of life during the postreproductive phase. This depends upon whether the presence of oldsters redounds to the benefit of the younger members of the society. All these genetic and evolutionary factors work toward increased variability during the postreproductive years. It is a plausible and testable working hypothesis that genetic differences that cause relatively minor variations during childhood and adult life may force the processes of senescence to take divergent paths. For a long time the possession of different blood antigens seemed to be adaptively inconsequential. The blood groups seemed to be "neutral" traits. Recent work by a number of investigators suggests, however, that some forms of duodenal and stomach ulcers, and perhaps also susceptibility to certain carcinomas, may occur more often in the carriers of some blood groups than of others. Since these diseases affect mainly the postreproductive and late reproductive ages, their influence on the selective values of the genotypes that are relatively immune or susceptible to them may not be great. However, this does not make them any less important from the standpoint of public health. The same may be true of the constitutions that predispose to excessive deposition of fat and to obesity and, incidentally, to certain disorders of the circulatory system.

Only a brief consideration can be given here to the tremendous problem of deviations from the adaptive norm, that is, to what are usually referred to as hereditary defects and diseases. Human populations, like populations of *Drosophila*, carry genetic loads of more or less sharply deleterious genetic variants. These loads are constantly being reduced by natural selection and replenished by the mutation process. Certain factors operative in advanced industrial societies, especially the misuse of X rays and of atomic energy, increase the mutation rates, and therefore the genetic loads. It is very probable that man, like *Drosophila*, has also many genetic variants that increase the fitness of the heterozygotes, but that are injurious, or even lethal, in homozygotes. The genetic load manifests itself on the phenotypic level in the occurrence of congenital and constitutional defects and diseases.

The fact that the deviations from the adaptive norm afflict the prereproductive and reproductive stages is crucial from the standpoint of evolutionary genetics. This does not mean that hereditary diseases are, as the man in the street usually supposes, incurable. The deviants from the adaptive norm make their carriers develop defective or morbid phenotypes in the normal environments, that is, in the environments in which the reproductive norm of its species results in high fitness. In other words, the deviations from the adaptive norm yield developmental paths that are deficient in homeostatic buffering against normal environmental variations during the prereproductive and reproductive years.

Some of the deviant genotypes are nevertheless able to produce satisfactory fitness if placed in special environments. Thus, genetically determined diabetes mellitus is "cured" by insulin injections. It is cured in the sense that insulin injections modify the phenotype in the direction of high fitness. Insulin certainly does not alter the diabetic genotype. The lesson to be drawn from this is that different human genotypes may require different environments for the optimal development of the phenotypes that they produce. Some genotypes require for this purpose special environments that can be provided only with the aid of modern medicine and technology. To put it differently, the deviant genotypes lack the environmental versatility of the adaptive norm.

Certain experiments made in *Drosophila* are interesting in this connection. Reference has been made above to the fact that flies homozygous for chromosomes sampled from natural populations are very often weak, sterile, or even inviable. The flies in their natural habitats are usually heterozygotes, the two chromosomes of each pair differing in their gene contents. Experiments have now been made exposing the same homozygotes and heterozygotes to different environments. Most heterozygotes proved to be highly and uniformlly viable in all the environments tested. Their homeostatic buffering is well developed. The behavior of the homozygotes was quite variable. Some few of them showed very high viabilities in certain environments, in fact higher than the average of the heterozygotes. However, not one of the homozygotes tested was "supervital" in all the environments. Normal or superior viability in one environment usually contrasted with poor viability in other environments. Many homozygotes were, of course, weak in all the experimental environments. Whether environments could be found in which these homozygotes would show vigor is a moot point. What seems to be characteristic of homozygotes in *Drosophila*, and possibly in other sexually reproducing and cross-fertilizing organisms, is not so much a low fitness as a deficient homeostatic buffering. Homozygotes are more often poorly viable than heterozygotes precisely because the latter do and the former do not possess environmental versatility.

Just why should heterozygosis so often improve and homozygosis weaken the homeostasis is one of the most intriguing problems of population genetics. Wallace recently discovered that the viability of inbred homozygotes in *Drosophila* is slightly, but significantly, improved by having one of the chromosomes of a pair undergo a mild X-ray treatment. The full import of this discovery is not yet clear. It appears to mean that, under certain conditions, the average viability is enhanced by heterozygosis for newly induced mutations (here a caveat: This finding certainly does not mean that X-ray-induced mutations tend to be useful!). It is obviously urgent to investigate the effects of heterozygosis for newly induced mutations on the viability of outbred lines in *Drosophila* and in other sexual cross-fertilizing forms, to inquire into the effects of such mutations on the viability of regularly self-fertilizing species, and to compare the effects of these mutations when heterozygous and when homozygous. In this field we may be on the threshold of major discoveries.

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